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Investigating the role of miR-21 in adult neurogenesis

Investigating the role of miR-21 in adult neurogenesis

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Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs that act as post-transcriptional regulators and play important roles in neurodegenerative diseases and brain disorders (Nelson et al. 2008). MiR-21, a miRNA that is dysregulated in cancers including glioblastomas, targets cellular processes including cell proliferation and apoptosis (Krichevsky & Gabriely 2009). MiR-21 has been shown to be upregulated following traumatic brain injury and spinal cord injury; this upregulation has been postulated to reduce lesion size, enhance cell survival and confer better neurological outcome (Ge et al. 2014; Hu et al. 2013). Due to its effects on cell proliferation and survival, miR-21 was speculated to play a role in adult neurogenesis in the mammalian brain. The effect of altering miR-21 levels on the cell fate of newborn neurons in the adult hippocampus was investigated using transgenic mice that globally either overexpress miR-21 (miR-21 OE) or lack miR-21 (miR-21 KO). First, increased neurogenesis in the dentate gyrus (DG) of miR-21 OE mice was detected, while miR-21 KO mice showed reduced neurogenesis in the same area. Transgenic mice lacking miR-21 (miR-21 KO) demonstrated impairment in learning and memory in the Morris water maze task. Mir-21 KO mice also showed reduced neurogenesis in the subventricular zone. To further understand the pathways that are involved in miR-21 regulation in the adult brain, miR-21 targets were investigated experimentally and using bioinformatics prediction tools. These results suggest that miR-21 plays an important role in regulating adult neurogenesis and learning behaviour. Overall, this is the first study to investigate miR-21 altered expression role in the adult normal brain. Linking

miR-21 role in this study to increased miR-21 levels in the brain and spinal cord after injury, will help to identify possible therapeutic strategies for treating traumatic injuries and neurodegenerative diseases.

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: ……. Date: ……

Table of contents

List of Figures

List of abbreviations

- 3’ untranslated region (3’UTR)
- 4,5-Bisphosphate (PIP2)
- 4,5-Trisphosphate (PIP3)
- 5’-Bromo-2-deoxyuridine (BrdU)
- Acetylcholine (Ach)
- Acidic nuclear phosphoprotein 32 family member A (ANP32A)
- Alzheimer’s disease (AD)
- Amyloid β (Aβ).
- Angiotpoietin-2(Ang-2)
- Ankyrin Repeat Domain 46 (ANKRD46)
- Argonaut 2 (Ago2)
- Argonaute protein (Ago)
- B-cell lymphoma 2 (BCL2)
- B-cell translocation gene 2 (BTG2)
- Bcl-2-associated X protein (BAX)
- Bone morphogenetic protein receptor type 2 (BMPR2)
- Bone morphogenic protein receptor (BMPR)
- Brain-derived neurotrophic factor (BDNF)
- cAMP response element binding (CREB)
- Cell division cycle 25 protein (CDC25A)
- Chordin-like 1 (Chrd-1)
- Coding sequence (CDS)
- Cornu ammonis (CA)
- Cyclin-dependent kinase 2-associated protein 1 (CDK2AP1)
- Dentate gyrus (DG)
- Doublecortin (DCX)
- Drosha and DiGeorge syndrome critical region gene 8 (DCGR8)
- Dulbecco's Modified Eagle's Medium (DMEM)

- EH Domain Containing 1 (Edh1)
- Fas ligand (FasL)
- Fluorescent in situ hybridization (FISH)
- Forkhead box O3A (FOXO3A)
- GABAA receptors containing the δ subunit (δGABAAR)
- Gamma aminobutyric acid (GABA)
- Gene Ontology (GO)
- Glial fibrillary acidic protein (GFAP)
- Granule cell layer (GCL)
- Green fluorescent protein (GFP)
- Human immunodeficiency virus (HIV)
- Huntington's disease (HD)
- In situ hybridization (ISH)
- Insulin-like growth factor 1 (IGF-1)
- Interleukin 6 (IL6)
- Interleukin-12 subunit alpha (IL12A)
- Jagged 1 protein (JAG1)
- Kyoto Encyclopedia of Genes and Genomes (KEGG)
- Long term potentiation (LTP)
- LRR Binding FLII Interacting Protein 1 (LRRFIP1)
- Messenger RNA (mRNA)
- Methylazoxymethanol acetate (MAM)
- MicroRNA recognition elements (MRE)
- MicroRNAs (miRNAs)
- Mitogen-activated protein kinase (MAPK)
- Molecular layer (ML)
- Morris Water Maze (MWM)
- MutS homolog 6 (MSH6)
- Myocyte enhancer factor 2C (MEF2C)
- Myristoylated alanine rich protein kinase C substrats (MARCKS)

- Nerve growth factor (NGF)
- Neural progenitor cells (NPCs)
- Neural stem cells (NSCs)
- Neuroectodermal stem cell marker (Nestin),
- Neurogenesin (Ng1)
- Neuronal nuclear antigen (NeuN)
- N-Methyl-D-aspartate (NMDA)
- Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)
- Olfactory bulb (OB)
- Orbitofrontal cortex (OFC)
- Parkinson's disease (PD),
- Partner of Drosha (Pasha)
- Pellino homolog 1 (PELI1)
- Peroxisome proliferator-activated receptor alpha (PPARA)
- Phosphatase and tensin homolog (PTEN)
- Platelet derived growth factor (PDGF)
- Post BrdU injection (PBI)
- Precursor microRNA Pre-miRNA
- Primary microRNA Pri-miRNA
- Programmed cell death 4 (PDCD4)
- Protein kinase B (AKT)
- Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)
- Reversion-inducing-cysteine rich protein with Kazal Motifs (RECK)
- Ribonucleic acid (RNA)
- RNA Polymerase II (RNA Pol II)
- RNA-induced silencing complex (RISC)
- Smad 7 protein (Smad7)
- Sonic hedgehog (Shh)
- Spinal cord injury (SCI)
- Spinocerebellar ataxia (SCA)

- Sprouty homolog 1 (Spry1)
- Sprouty homolog 2 (Spry2)
- SRY (sex determining region Y)-box 2 (SOX2)
- SRY-box containing gene (Sox9)
- Sub granular zone (SGZ)
- Sub ventricular zone (SVZ)
- SWI/SNF related, matrix associated, actin dependent regulator of Chromatin, subfamily a, member 4 (SMARCA4)
- TAR binding proteins in mammals (TRBP)
- Thyroid Hormone Receptor Beta (THRB)
- Tissue inhibitor of metalloproteinases 3 (TIMP3)
- Transforming growth factor, beta 1 (TGF-β1)
- Transforming growth factor-beta receptor type 2 (TGFBR2)
- Transmembrane protein 49 (Tmem49)
- Traumatic brain injury (TBI)
- Tropomyosin1 (TPM1)
- Tumour protein 63 (TP63)
- Vacuole Membrane Protein 1(Vmp1)
- Wild type (WT)

List of Tables

Chapter 1 Introduction

1.1 Background

Adult neurogenesis is the process of generating new neurons from stem cells located in discrete areas within the adult brain. These newly generated neural precursor cells (NPCs) then migrate and differentiate into all the major neuronal lineages: neurons, astrocytes and oligodendrocytes that mature to become fully integrated in the existing neural circuit. This neuroplasticity phenomenon is restricted to two main areas in the adult brain: the sub-granular zone (SGZ) within the hippocampal dentate gyrus (DG) and the sub-ventricular zone (SVZ) within the lateral ventricles. The regulation of this neuroplasticity process is controlled by a precise combination of intrinsic and extrinsic factors that send molecular signals within the microenvironment that these NPCs are embedded in. The idea of a self-renewal process in the adult brain is contradictory to the long-held belief that no neurons are generated in the brain after postnatal development, and it still faces scepticism. This is due to the lack of clear understanding of the importance of adult neurogenesis and its function in a healthy normal brain.

In this chapter, I will present background knowledge related to the field of adult neurogenesis, including the development of the concept itself. Next, I will move on to describe the process of neurogenesis, with a focus on the two brain regions associated with adult neurogenesis, its regulation and the functional significance. This will be followed by a description of how the impairment of these processes can result in the development of diseases. I will briefly cover molecular mechanisms regulating adult neurogenesis with a focus on microRNAs, specifically microRNA-21. I will

explain the reasons for studying this microRNA, implications associated with its function, and its role in adult neurogenesis.

1.1.1 Historical background

It has long been believed that the adult mammalian brain has no capacity to generate new neurons. The father of modern neuroscience Ramon y Cajal stated that:
"In the adult centres, the nerve paths are something fixed, finished, immutable: everything may die, nothing may be regenerated." (Ramón y Cajal, 1928)

This long-held belief was supported by philosophical ideas that if new neurons were added to the existing neurons this would lead to the loss of reasoning and therefore instability in the individual. Additionally, having millions of neurons with their complicated morphology and many connections in the mature terminally differentiated post-mitotic state indicates that cells are no longer able to undergo mitosis (Aranda-Anzaldo & Dent 2017). However, adult neurogenesis was discovered in the 1960s by Altman using 3H-thymidine autoradiography to label the nuclei of proliferating cells. This study was the first to provide evidence of "undifferentiated cells" in the DG of adult rat brain (Altman & Das 1965). Since then, studies have demonstrated the generation of new neurons in a variety of brain structures such as the olfactory bulb, the hippocampus and the cerebral cortex (Stanfield & Trice 1988). More recently, the use of 3H-thymidine labelling and Bromodeoxyuridine (BrdU) with cell specific markers for immunohistochemistry, in combination with advances in microscopy, has confirmed the identity of newly generated neurons (Boseret et al. 2007). Since the discovery of adult neurogenesis,

the field has grown dramatically in terms of research and publications. The process of adult neurogenesis is unique, as developmental neurogenesis establishes the cortical layers, whereas adult neurogenesis adds to the pre-existing cortical layers. Since its discovery, roles for adult neurogenesis have been implicated in different neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Spinocerebellar ataxia (SCA). However, the link between adult neurogenesis and disease progression needs further investigations to identify the cellular and molecular mechanisms involved (Zhao et al. 2008).

1.2 Neurogenic niches in the adult brain

NPCs can be generated *in vitro* from any part of the adult nervous system, however, adult neurogenesis only occurs in two main areas of the adult mammalian CNS: the SVZ within the lateral ventricles and in the DG within the hippocampus (Reynolds & Weiss 1992). Cells isolated from these areas can give rise to all three neural lineages (neurons, astrocytes and oligodendrocytes) and undergo self- renewal (Bonaguidi et al. 2008) (Figure 1-1). Therefore, it was proposed that the microenvironment of these areas has some factors that encourage the differentiation of these NPCs and their integration, and failure to provide these factors prevents the development of new neurons in the adult brain (Ashton et al. 2012a; Apple et al. 2016)

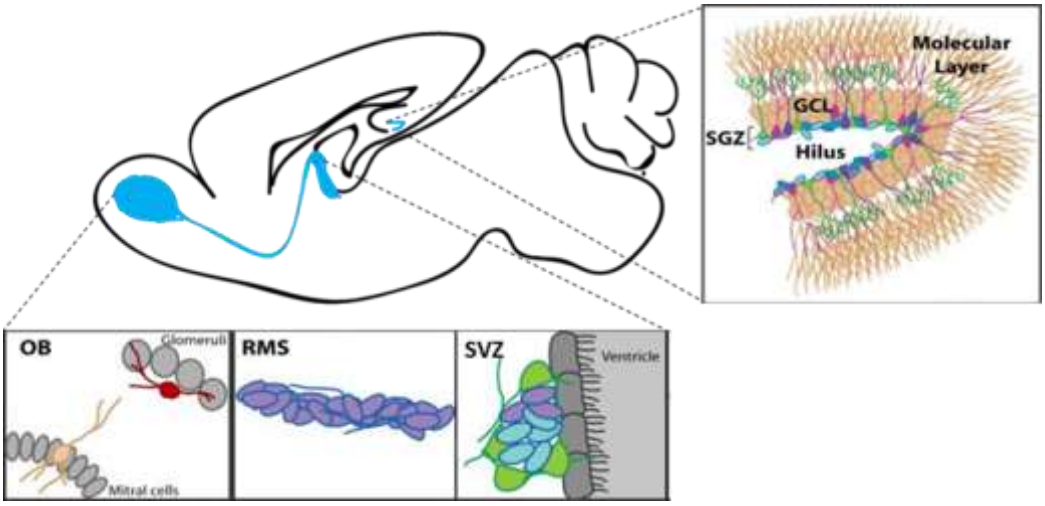


Figure 1-1: Neurogenesis niches in the adult mouse brain.

Sagittal image of the adult mouse brain representing the two neurogenic regions: the DG within the hippocampus, and the SVZ within the lateral ventricles. In the DG, neural progenitor cells extend along the SGZ, where they divide to give rise to the neuroblasts and then immature and mature neurons. Mature neurons send their dendrites through the granule cell layer (GCL) to the molecular layer to receive input from the entorhinal cortex. The SVZ neural stem cells (type B cells) lining the wall of lateral ventricles (LV) give rise to neuroblasts that migrate along the rostral migratory stream (RMS) to reach the olfactory bulb (OB) where they differentiate. Newborn neurons in the OB receive inputs from olfactory sensory neurons to form excitatory synapses and inhibitory interneurons. Figure reproduced from (Johnson et al. 2009).

The importance of the microenvironment in influencing the regeneration ability in the areas of the adult brain has been highlighted by results obtained from transplantation studies. *In vitro*, both embryonic and adult NPCs show regenerative capacity. However, when these cells were grafted in anti-neurogenic areas such as in adult spinal cord, they lost their ability to generate neurons and instead formed astroglia (Hofstetter et al. 2005; Enzmann et al. 2005), even with the use of neuron reprogramming factors such as Pax6, Ascl1, and Neurog2 that are found to be expressed in the SVZ, neonatal cortex and stratum (Grande et al. 2013). In fact, the

only regions in the adult brain that the transplanted cells could produce neurons were the DG and SVZ (Shihabuddin et al. 2000). NPCs from the spinal cord when transplanted into adult rat DG with fibroblast growth factor (FGF2) differentiated into neurons and astrocytes further confirming the multipotentiality and self-renewal abilities of these NPCs (Shihabuddin et al. 2000). These studies demonstrate the importance of the environment surrounding the NPCs; it is likely that the molecular components of the microenvironment in the neurogenic niches of the adult CNS are highly influenced by local secreted cytokines, hormones and the extracellular matrix, and this causes the occurrence of neurogenesis over gliogenesis.

In the adult CNS there are several similar components involved in the regulation of neurogenic niches including blood vessels, astrocytes, microglia, neurotransmitters and extracellular matrix (Silva-Vargas et al. 2013; Bracko et al. 2012). However, there are other specific components that regulate each of the neurogenic regions (Zhao et al. 2008). In both regions, the process of neurogenesis starts with the activation of the quiescent population of NPCs to generate proliferating cells to give rise to neurons (Kempermann et al. 2003). The factors involved in the activation of NPCs from the quiescent state are still under investigation as well as the process and number of asymmetric divisions before differentiation (Bonaguidi et al. 2012).

In the DG, a number of factors have been shown to regulate the transition from quiescent state to the active more proliferative state including: bone morphogenetic protein (BMP), involved in the regulation of the balance of proliferation and differentiation; and wingless/integrated (Wnt) which coordinates with Sox2 to promote the proliferation and then the differentiation of NPCs by the regulation of

Neurod1 protein (Kuwabara et al. 2009; Sahni et al. 2010). Astrocytes within the DG play an important role in regulation of the DG niche. Astrocytes secret ephrin-B2, which in turn activates the expression of β-catenin *in vitro* and *in vivo* to promote NPCs differentiation (Ashton et al. 2012). Additionally, astrocytes release Wnt and this leads to increased neurogenesis in the adult DG though the regulation of β-catenin pathway (Lie et al. 2005). Microglia are another important component in the DG neurogenic niche. They are the primary immune surveillance cells within the CNS, and they survey the function of neurons, astrocytes, oligodendrocytes among other components to maintain neurogenic niche (Wake et al. 2013; Spiller et al. 2018). Additionally, microglia maintain the NPCs population by rapidly removing apoptotic adult newborn DG cells by phagocytosis (Sierra et al. 2010).

Adult stem cells reside in the resting quiescent state for prolonged periods of time, which is a state maintained by different signalling pathways. In the SVZ, the transition from the quiescent state to the active state of stem cells is regulated by endothelial receptors ephrinB2 and Jagged1, which are secreted from blood vessels to inhibit cell cycle reentry and suppress differentiation, thus maintaining resting stem cell population in the quiescent state (Ottone et al., 2014). Another study showed that platelet derived growth factor (PDGF), which is expressed by the endothelium cells of the SVZ, is involved in the regulation of self-renewal of adult NPC *in vivo* and *in vitro* (Notari et al. 2006).

1.2.1 Neurogenesis in the adult SVZ

The SVZ has the largest population of proliferating transit-amplifying cell (type C cells) in the adult brain in most mammals (Altman 1969; Doetsch et al. 1997). These

cells reside within a layer along the walls of lateral ventricles. New progenitor cells that are destined for differentiation into neurons migrate through the rostral migratory stream and reach the olfactory bulb (OB), where they spread radially and differentiate into interneurons. Neurogenesis continues as the cells differentiate into distinct types of olfactory neurons (Carleton et al. 2003). Half of those newborn cells survive long-term, and most of them integrate into functional circuits (Sakamoto et al. 2014). In addition, the SVZ is the birthplace of the oligodendrocytes in both normal and diseased brains, which makes the SVZ region interesting for the study of neurogenesis and the generation of oligodendrocytes. SVZ neurogenesis is a process that continues throughout life, with thousands of new cells added to the OB daily, and given the stable nature of the adult SVZ cytoarchitecture this has led to intensive study of this region (Obernier et al. 2014).

The adult mouse SVZ comprises four different cell types ordered by their position in relation to the lateral ventricles: ependymal cells, Types B, C and A cells, which can be defined by their morphology and associated markers such as Glial fibrillary acidic protein (GFAP), SRY (sex determining region Y)-box 2 (SOX2) and neuroectodermal stem cell marker (Nestin), Neuronal migration protein doublecortin (DCX) and neural nuclear antigen NeuN. The ependymal cell layer is made up of multiciliate epithelial cells that can function as stem cells (Doetsch et al. 1997). This layer is not completely contiguous along the ventricles with B cells sending thin cellular processes through ependymal cells as shown in Figure 1-2. Type B cells have astrocytic morphology and express the astrocyte marker GFAP and function as self-renewing NPCs that can be activated to generate transit amplifying cells (type C)

within the SVZ (Doetsch et al. 1997). Type B cells contact with type C cells and type A cells (migrated neuroblast). type C cells are a group of rapidly dividing immature cells that function as an intermediary between Type B cells and A cells. These rapidly dividing cells can be reprogrammed *in vitro* by culture conditions such as ubiquitination system E3-ubiquitin ligase Huwe1 (HECT, UBA, and WWE domain–containing 1) to gain self-renewal properties and function as NPCs, much like the B cells (Ponti et al. 2013). Type A cells form chains ensheathed by SVZ astrocytes, which support the migration of neuroblast to the OB. The migrating A cells migrate through the RMS for up to 5mm away from the SVZ, to reach the OB. When migrating neuroblasts reach the OB, they depart from the tangential orientation of the RMS and migrate radially. Radially migrating neuroblasts are attracted to the appropriate OB layer, including the glomerular layer (GL) and the granule cell layer (GCL), where they differentiate into inhibitory interneurons and contribute to the exciting neural circuit (Lledo & Valley 2016).

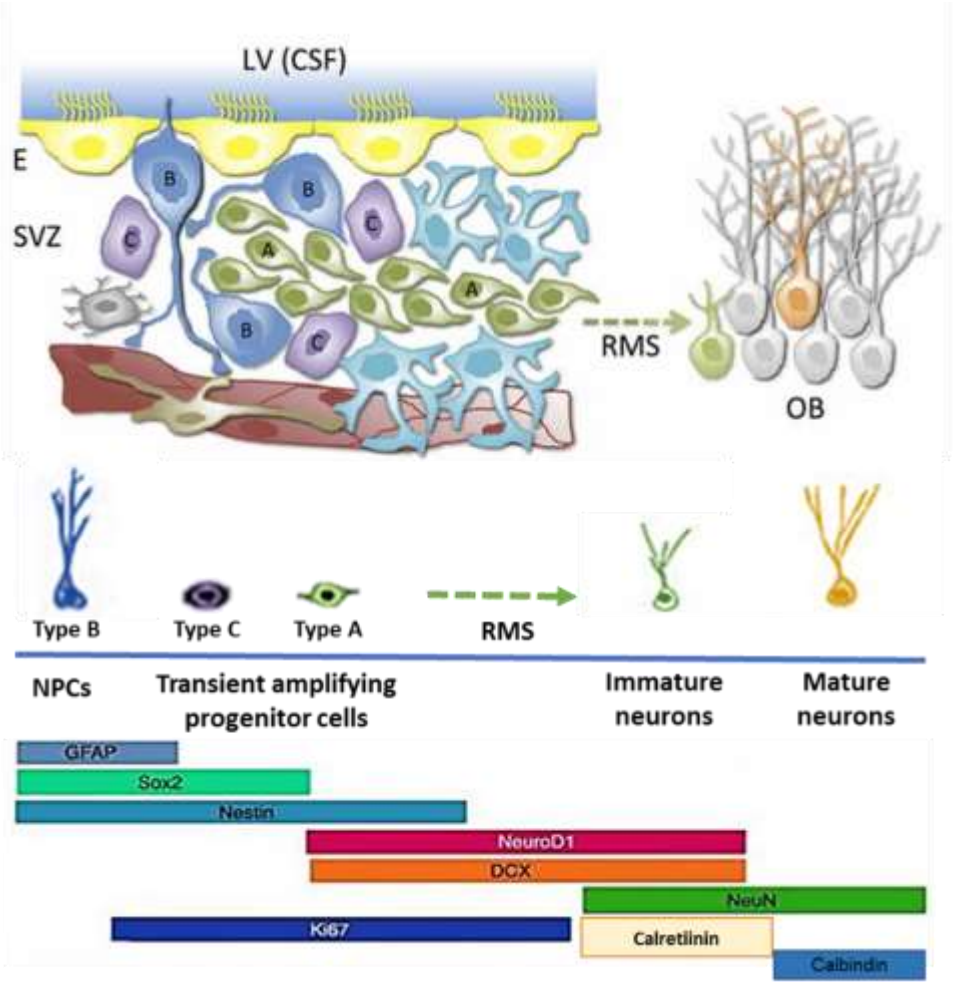


Figure 1-2: Cytoarchitecture of the SVZ and associated cell markers.

The SVZ resides underneath the ependymal layer (E) of the LV that is filled with cerebrospinal fluid. It is composed of type B quiescent cells (NPCs), which can be activated to generate transit amplifying cells (type C) and then generate neuroblasts (type A). Neuroblasts migrate through the rostral migratory stream (RMS) to reach the OB where they differentiate to immature granule neurons then mature to become mature granule neurons. Figure adapted from Bátiz et al. (2016).

1.2.2 Adult neurogenesis in the adult hippocampus

Of the two regions involved in adult neurogenesis, the hippocampus has attracted more attention due its association with higher cognitive functions such as learning, memory and neuronal plasticity. In humans, adult neurogenesis is only found in the hippocampus, which makes it an interesting model for study (Kempermann et al. 2015). Defects in hippocampal neurogenesis have been linked to the development of several psychiatric disorders, such as depression and anxiety (Apple et al. 2016). It has also been suggested that people with glioma can suffer from depression due to a lack of hippocampal adult neurogenesis related to chemotherapy treatment (Egeland et al. 2017). In addition, several genes that are associated with aggregates in neurodegenerative diseases such as α-synuclein, presenilin-1, tau, huntingtin are also liked to neurodegenerative diseases including Parkinson's disease, Huntington's disease and Alzheimer's (Worlitzer et al. 2012).

Adult-born dentate granule neurons reside within the bottom layer of the GCL where they go through several developmental processes to reach full integration in the hippocampal circuit. Radial glia-like cells (RGLs) or type 1 cells are the population of proliferating cells within the subgranular zone (SGZ) of the dentate gyrus (DG). They give rise to the intermediate progenitor cells (IPCs) or type 2 cells, which are similar to the transient amplifying cells in the SVZ region. These type 2 cells can give rise to neuroblasts (type 3) that subsequently migrate a short distance within the granule cell layer and differentiate into immature dentate granule neurons (Figure 1-3). This process for RGLs to become mature neurons and develop dendrites into the

molecular layer and axons that connect to target cells in the hilus and pyramidal cells in the cornu ammonis (CA) takes 4-8 weeks.

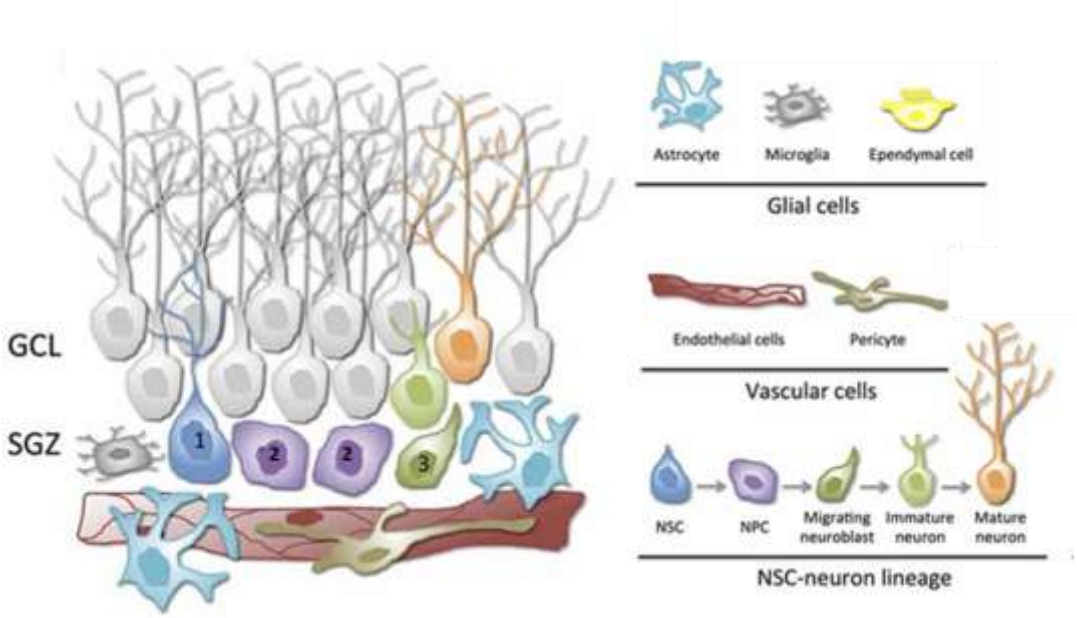


Figure 1-3: Cytoarchitecture of the sub granular zone and dentate granule layer of the adult hippocampus.

The figure illustrates the development of dentate gyrus granule cells from stem cells to mature neurons. New neurons developed from radial glial cells (type 1) and the rapidly amplifying (type 2) neural progenitor cells. Type 1 and 2 cells differentiate into neurons and develop dendrites and axonal projections. New neurons receive excitatory input from the perforant cortical path, and they approach a fully mature neuron anatomy and physiology by week 4-8. Figure taken from Goldman & Chen (2011).

1.2.2.1 The hippocampal circuit

Although it is known that neurogenesis occurs in the hippocampus, it needs be clarified that neurogenesis occurs mainly in GCL of the DG from which granule cells (GCs) project their axons to the CA3 area of the Ammon's horn of the hippocampus (Kuhn et al. 1996). In the DG, new cells differentiate mainly into immature neurons that represent a small fraction (<0.5%) of the total mature neuronal population in

rodents. In humans, it has been estimated that about 700 new-born neurons are added daily to the DG. This number is relatively small at any one time compared to the billions of neurons in the human brain; however, neurons within the population are continuously exchanged and by the age of 50 a complete set of neurons has been replaced since the individual's birth (Ernst & Frisén 2015). Newly generated cells in the SGZ migrate a short distance into the granular cell (GC) layer of the DG and integrate into the existing circuitry of the hippocampus. The new neurons mature to have similar morphology and function as neurons born during development, despite the fact that their intrinsic properties and connectivity are different to those generated during early development (Kempermann et al. 2003; Laplagne et al. 2006). Newborn neurons in the DG and the OB have enhanced synaptic activity due to their hyper excitability, which facilitates their integration into the local network and contributes to information processing, reflected in specific type of learning and memory functions such as pattern separation (Leutgeb et al. 2007) and spatial distribution (Lledo et al. 2006a).

The process of hippocampal adult neurogenesis generates one type of neuron: granule cells, which receive excitatory glutamatergic input from the entorhinal cortex and project to the dendrites of CA3 pyramidal cells in the hippocampus through the mossy fibres; CA3 neurons send axons to the CA1 pyramidal cells, which then project to the subiculum and back to the entorhinal cortex. Additionally, there is a direct entorhinal network connection, whereby the entorhinal cortex can bypass the DG to project to the CA3 and CA1 pyramidal cells directly for information processing (Leutgeb et al. 2007; Nakazawa et al. 2004).

1.3 Adult neurogenesis and aging

The adult brain's ability to generate new neurons in the SGZ and in the SVZ is fascinating. In the SGZ, NPCs differentiate to produce either neurons or astrocytes and in the SVZ, NPCs differentiate in the OB to produce two types of inhibitory interneurons: periglomerular and granule cells; and corpus callosum oligodendrocytes (Lim & Alvarez-Buylla 2016; Bond et al. 2015). In both regions, newly generated neurons mature to become functional neurons that are integrated structurally into a pre-existing network. Although the process of adult neurogenesis is regulated by many factors, aging has been designated as one of the most negative regulators of adult hippocampal neurogenesis (Kuhn et al. 1996; Kempermann 2015). Throughout life, a general progressive reduction in the rate of generating new neurons occurs, as aging is associated with reduced neurogenesis. This reduction in adult neurogenesis can differ among different types of mammals, as the process can be largely impaired in aged rodents, but still maintained in humans (Ernst & Frisén 2015). Although aging is not a disease, it is associated with cognitive impairments including loss of neurons and connections, reduction of neurogenesis and degradation of hippocampal circuits (Kempermann 2015). Decrease in adult neurogenesis in aged OB is also observed and this may be linked to impaired olfactory discrimination skills (Enwere et al. 2004).

1.3.1 Adult neurogenesis and neurodegenerative diseases

Neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD) are associated with progressive decline of specific neuronal populations, for example the dopaminergic neurons in the substantia nigra for PD, cholinergic neurons in the forebrain for AD and the medium spiny

neurons in the striatum for HD (Maciotta et al. 2013). Since the adult brain can generate new neurons, it would be interesting to study the possibility of directing newly generated neurons to migrate to the affected areas and differentiate into specific type of neurons that have died. In addition, neurodegenerative diseases are linked to aging, so it might be that the progression of these diseases is a consequence of reduced adult neurogenesis or its inhibition (Winner & Winkler 2015).

Adult neurogenesis is comprised of three main steps: proliferation of NPCs that results from asymmetric cell division of stem cells, migration of a newborn cell to its final location in the brain and survival of the matured neuron. Therefore, it is important to address the molecular mechanisms associated with all these steps and study if there are any changes occurring in the case of any possible diseases and/or trauma. For example, in PD, the levels of α-synuclein are increased and this leads to reduced neurogenesis and increased apoptosis in the SVZ (Regensburger et al. 2014). As neurogenesis cannot occur in the substantia nigra (area affected by PD) either in intact or in nigral lesions (Gashab & More, 2005; Steiner et al., 2006), the lost dopaminergic neurons in the substantia nigra are not replaced by new cells.

In the case of HD, several pieces of evidence suggest that neurogenesis is increased in the striatum (Tattersfield et al. 2004; Curtis et al. 2003), while others suggested that HD results in reduced neurogenesis in the SVZ-derived neuroblasts in the striatum of human and animal adult brain (Lazic et al. 2004; Ernst et al. 2014; Kandasamy & Aigner 2018). The development of rodent Huntington's disease models has helped to explain the disease pathology and the molecular mechanisms involved as well as identify possible therapeutic interventions (Mangiarini et al. 1996; Yang et

al. 2008). The Huntington's disease mouse model (R6) was generated by the insertion of a cytosine-adenine-guanine (CAG, translated into glutamine), in the first exon of the human huntingtin (HTT) gene. This model developed HD neurological features including uncontrolled body movement, weight loss, cognitive impairment and death (Mangiarini et al. 1996). Several studies have found reduced proliferation in the DG of R6 transgenic mice. BrdU+ and Ki67+ cells, which are markers for proliferating cells, were reduced, as well as neurogenesis as indicated by reduced DCX+ and NeuN+ cells (van der Borght & Brundin 2007). These results were found in the adult DG alone, as the SVZ did not have reduced proliferation (Gil-Mohapel et al. 2011).

AD is a common neurodegenerative disease that results from the loss of synapses in the frontal cortex and limbic system and it involves degradation in synaptic plasticity, accumulation of neuro-fibrillary tangles (Tau) and deposition of amyloid β (Aβ). Studies on the human brains of AD patients and on experimental models of AD have found reduced hippocampal neurogenesis in AD and increased proliferation of glial cells (Boekhoorn et al. 2006). AD mouse models display a severe reduction of proliferating cells and neurogenesis in the DG by 75% at the age of 9 months, progressing to complete ablation by 12 months, which was correlated to an increased accumulation of Aβ plaques (Yu et al. 2009). In addition, Aβ accumulation results in increased apoptosis, that leads to the activation of pathological processes such as mitochondria dysfunction and reduced NPC migration (Glabe & Kayed 2006).

The association between decreased adult neurogenesis and neurodegenerative diseases such as PD, HD and AD suggests that disease mechanisms may be involved in the modification of the adult neurogenesis process. Although limited knowledge is

available in relation to neurodegeneration and adult neurogenesis, further understanding of the mechanisms that contribute to the enhancement of adult neurogenesis and/or control of neuronal loss can serve to improve cognitive conditions in affected patients.

1.4 Functional significance of adult neurogenesis

Neurogenesis is a process that involves generating newborn neurons that are able to interact with the existing neural circuit and produce electrophysiological activities. Adult neurogenesis is observed in the postnatal period and throughout adulthood in the two restricted regions: the SVZ and the SGZ, and it is still unclear why adult neurogenesis only persists in these areas of the brain that already have functional networks. It is possible that newly generated neurons perform specific tasks that cannot be fulfilled by mature neurons. Additionally, it seems that adult neurogenesis is not a restorative process, but an adaptive process that enables the brain to engage with a rapidly changing environment (Zhao et al. 2008; Gonçalves et al. 2016). These aspects are going to be further addressed.

The olfactory system serves to detect chemical cues from the environment; this function is performed by olfactory receptor neurons that send axons to the glomerulus of the olfactory bulb. Within the olfactory bulb, complex neural circuitry consisting of excitatory and inhibitory interneurons within the external plexiform layer, mitral cell layer and granule cell layer processes the olfactory information and sends an output to the amygdala, the orbitofrontal cortex (OFC) and the hippocampus where it contributes to emotion, memory and learning (Aimone et al. 2011). Newly generated interneurons are integrated into this neural circuitry however, the direct result of this

integration is still unclear (Lledo et al. 2008). About 94% of the newly generated neurons that reach the OB are granule cells, while the remaining cells are astrocytes and periglomerular cells (Lledo & Valley 2016). Granule cells are connected to hundreds of mitral cells, which in turn send their synapses to the pyramidal cells in the piriform cortex. Granule cells can be activated by mitral cells to produce GABAergic inhibition in the same neurons, nearby granule neurons or mitral cells. The connection between adult newborn interneurons and the rest of the OB and brain is important for the actions and survival or these new neurons (Hack et al. 2005). One of the suggested functions of the newborn granule neurons is to improve plasticity of the neuronal networks, as it has been found that sensory deprivation can result in decreased spine density and dendritic length of newly generated interneurons (Saghatelyan et al. 2005). Thus, cell turnover may be important for learning and distinguishing newly generated information. A second function is that newly generated interneurons can work to adapt to novel sensory functions (Lledo & Saghatelyan 2005). This was supported by loss of function experiments, as small doses of irradiation for 2-4 days resulted in decreased proliferation and neural differentiation, and increased apoptosis in dividing cells (Santarelli et al. 2003). However, sensory afferents are subject to continual renewal, as they have a restricted lifespan and have high sensitivity to the odorant space. It was found that new neurons in the OB have longer life spans due to an increased survival rate (Lledo et al. 2005). Therefore, these findings suggest that learning based on sensory stimuli can affect adult neurogenesis in the OB.

Hippocampal neurogenesis has been linked to enriched environments and physical activity. Newly generated adult neurons can be beneficial for brain functions particularly spatial memory and pattern separation (from a behavioral perspective this is the ability to discriminate between similar memories). Immature neurons have a low activated threshold with higher responsive integration than mature neurons, and thus are capable of encoding memories from very similar stimuli (Clelland et al. 2009). Mature neurons are involved in encoding past events while new neurons are capable of encoding novel events, and the cooperation between both functions is important for generating more robust memories. Exposing an animal to enriched environments improves the animal's abilities in spatial tasks and increases the number of adult-born neurons in the DG (Kempermann et al. 1997). Although mechanisms involved in pattern separation are not clearly understood, behavioral experiments using inhibition approaches such as methylazoxymethanol acetate (MAM), a DNA methylating agent that ablates neurogenesis, along with performance on spatial discriminating tasks have suggested a strong relationship between adult neurogenesis and pattern separation (Shors et al. 2001). In that study, the inhibition of adult neurogenesis resulted in impaired pattern separation, but not the process of learning when stimuli were not separated in time (hippocampus independent learning task) (Shors et al. 2001). Furthermore, specific silencing of adult-born DG cells at 4 weeks of age but not their 2- or 8-week old counterparts after task acquisition using optogenetic techniques led to impaired memory recall (Zhuo et al. 2016). In addition, hippocampal adult neurogenesis is important for forgetting or unlearning memories in order to acquire new memories. Adult neurogenesis can serve as a mechanism of forgetting by modulating the hippocampal circuit to generate new memories, which

requires the removal of older memories in order to meet the capacity of working memory (Yau et al. 2015). Besides its role in learning and memory, adult hippocampal neurogenesis has been implicated in emotional regulation and anxiety as people treated from glioma developed depression, which has been suggested to be a consequence of chemotherapy and reduced NPC proliferation in the hippocampus (Dias et al. 2014; Sah et al. 2012; Snyder et al. 2011; Mateus-Pinheiro et al. 2013).

1.5 Adult neurogenesis in human

Many features of hippocampal adult neurogenesis are evolutionarily conserved in the mammalian brain (Altman and Das, 1965; Altman, 1969; Alvarez-Buylla and Nottebohm, 1988; Boldrini et al. 2018; Eriksson et al., 1998; Morest, 1970; Kaplan and Hinds, 1977; Goldman and Nottebohm, 1983; Pencea et al., 2001; Sawamoto et al., 2011). However, there are differences in SVZ neurogenesis between rodents, humans and primates. There is a large regression in the olfactory abilities of primates and humans, which is linked to reduced dependence on olfaction throughout evolution in primates and humans. Despite several studies that have demonstrated the presence of dividing neural progenitors in the SVZ, the addition of adult newborn neurons in the human OB is very limited (Ming & Song 2011; Sanai et al. 2011) and the existence of neuroblasts in the RMS of the adult human is extremely rare (Wang et al. 2011). Rodents, on the other hand, have retained olfactory behaviours and indeed, their sophisticated olfactory system is crucial to their day-to-day function such as, food finding, mating, exploring etc. However, another brain structure – the striatum - appears to gain more importance through the course of evolution in higher mammals and humans. The striatum is a forebrain structure located beneath the cortex

and is associated with motor behaviours and responses to reward. This importance is due to increased reliance on movement coordination, cognition and emotion in upper mammals (Koscik & Tranel 2012).

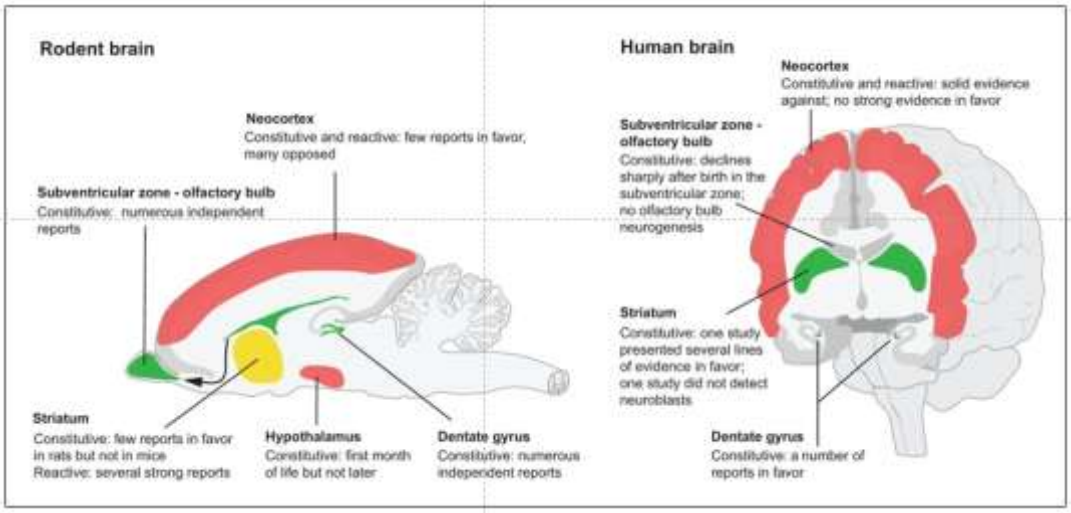


Figure 1-4: Comparison between adult neurogenesis regions in rodents and humans.

Green regions indicate regions where neurogenesis persists throughout life, red regions indicate places with neurogenesis in early developmental stages, but no evidence in adult stages, and yellow regions indicate places with injury-associated neurogenesis. Note that in human very little SVZ adult neurogenesis is detected, which is lost in the OB. Figure is taken from Magnusson & Frisén (2016).

1.6 Regulation of adult neurogenesis

In the adult brain, active neurogenesis is restricted to two main areas due to the unique niche structure in both the SVZ and the SGZ. The cellular and molecular composition of this neurogenic niche includes microglia, mature neurons, astrocytes, endothelial cells and ependymal cells, as well as transcription factors, extracellular matrix, hormones and non-coding RNAs including microRNAs, respectively.

Adult neurogenesis is largely regulated by the direct neurogenic niche which provides support to the neurogenesis process (Song et al. 2002). Although astrocytes are non-electrically excitable cells in the mammalian CNS, they are one of the major influencers in these neurogenic niches and regulate NPCs' proliferation, migration, neural fate commitment and survival (Song et al., 2002; Platel et al., 2010). Astrocytes play an important role in promoting proliferation and neuronal fate determination by secreting a number of factors that are involved in neuronal migration, synaptic formation and proliferation (Bazargani & Attwell 2016). Wnt family genes are expressed in astrocytes and are involved in the regulation of neurogenesis and fate specification by the control of underlying regulatory mechanisms (Barkho et al. 2006). Additionally, astrocytes express other secretory factors including cytokines and noggin-like proteins such as neurogenesin (Ng1), which are involved in neural differentiation and neurogenesis regulation (Barkho et al. 2006; Ueki et al. 2003). In addition, astrocytes release glutamate which can affect positively the survival of mature neuroblasts (Bazargani & Attwell 2016). The release of Noggin by ependymal cells (cells of astrocytic lineage) in the wall of lateral ventricles increases neurogenesis by inhibiting the function of BMP, which is a negative regulator of neurogenesis. Additionally, Noggin regulates the fate of NPCs by promoting neuronal differentiation (Lim et al. 2000; Ming & Song, 2011). Noggin function of neurogenesis regulation is by directing local stem cells toward the neuronal fate, in a process independent to the function of Wnt molecules (Song et al. 2002). Additionally, a previous study found that ephrin-B2, which is released from hippocampal astrocytes, is involved in the regulation of neurogenesis *in vivo*

(Ashton et al. 2012).These studies highlight the important role astrocytes play in the regulation of neurogenic niche.

Microglia are important contributors to the adult neurogenic niche. During the process of adult neurogenesis, only a small proportion of NPCs end up as mature neurons with the remaining undergo apoptosis, therefore activating the immune response (Ma et al., 2009). Microglia interact with local components of the adult neurogenic niches ensuring constant monitoring of the adult DG as they engulf the NPCs undergoing apoptosis, thus maintaining homeostasis (Nimmerjahn et al., 2005). It has been estimated that a limited subpopulation (~10%) of DG neurons are subject to exchange in rodents, while in human by the age of 50, the majority of hippocampal GCs are generated in the adult stage (Ernst & Frisén 2015). However, in the adult mouse SVZ, about 40% of the newly born neurons integrate and survive up to 18 months in mice (Winner et al. 2002). This indicates that despite efficient clearance of excess newly generated neural precursors by microglia, there is still a significant percentage of newly generated neurons that are maintained and functional in both the SVZ and DG.

1.6.1 Regulation by intrinsic factors

One of the reasons that adult neurogenesis is an interesting topic is the increasing number of stimuli that have been identified to be involved in the physiological and pathological regulation of adult neurogenesis. In general, any alteration of these stimuli can result in corresponding changes in cognitive functions, which refers to regulation rather than control of adult neurogenesis. This is because during early development genetic factors and intrinsic programs determine the fate of certain cell

types at a specific time point, which can be described as a control process for neurogenesis. However, during the adult stage although these genetic factors still exist, it is the interaction between the micro environment and the extrinsic factors which provides the major stimulus for the generation of new neurons and regulates adult neurogenesis. Therefore, in the adult stage development of brain structures is not the final goal of the process, but the balance between intrinsic control and extrinsic regulation, which determines the adaptation and plasticity of the newly generating neurons (Kempermann et al. 2015).

A number of signalling pathways are involved in regulating adult neurogenesis including local signalling pathways such as the Wnt proteins, BMPs, sonic hedgehog (Shh) and Notch signalling cascades. Trophic factors are involved in the stimulation of differentiation and survival of newly generated NPCs in both neurogenic regions. Brain derived neurotrophic factor (BDNF) is expressed by the dendrites of GCs in the DG and increases GABA input to neural precursors, which enhances the differentiation and maturation of NPCs into neurons (Waterhouse et al. 2012). The binding of growth factors to the neural stem cell surface receptors induces signalling cascades which can result in the stimulation of cell proliferation and/or differentiation. For example, fibroblast growth factor (FGF) is expressed during early developmental stages and in the adult CNS, where it is mainly expressed in the astroglial cells (Palmer et al. 1999). FGF is stimulated after brain injury and enhances cell repair and survival (Otto & Unsicker 1990). Additionally, it has been to regulate NPC proliferation. Lower concentrations of FGF (2ng/ml) is required to maintain neural differentiation and survival, while higher concentrations (20ng/ml) caused

enhanced proliferation in culture conditions (Gage et al. 1995). Similarly, other growth factors including transforming growth factor-β1 (TGF-β1), pigment epithelial growth factor (PEGF) and ependymal growth factor (EGF) stimulate the self-renewal state of stem cells and NPCs (Wachs et al. 2006; Ramírez-Castillejo et al. 2006).

In addition to growth factors, neurotransmitters such GABA, glutamate and acetylcholine (Ach) are also involved in the regulation of adult neurogenesis (Ming & Song 2011; Aimone et al. 2014). Newly generated neurons in the adult GCL send axonal projections through the hilus to the CA3 region in the hippocampus and receive glutamatergic and GABAergic synaptic inputs from the entorhinal cortex and local interneurons. GABA is one of the major inhibitory neurotransmitters that activates synaptic GABA receptors leading to either depolarization or hyperpolarization of the target cell, depending on the calcium (Cl^-) concentration across the neural membrane. GABA is released by local interneurons in the DG and is known to be involved in the regulation of cell proliferation, maturation and synaptic integration of the newly generated neurons (Ge et al. 2006). GABA inhibits NPC proliferation through regulation of epigenetic mechanisms that inhibits DNA synthesis. The excitatory activity of GABA promotes neuronal differentiation and survival (Aimone et al. 2014).

In addition, glutamate inhibits proliferation and differentiation of GCs and regulates the survival of newborn neurons in the adult DG through the regulation of NMDA receptor. Treatment with a NMDA receptor antagonist results in the stimulation of proliferation, while treatment with a NMDA agonist has the opposite effect in the adult DG (Cameron et al. 1995).

Epigenetic mechanisms such as methylations, histone modifications and non-coding RNAs including microRNAs, are important for gene expression coordination mechanisms of various aspects of adult neurogenesis (Sun et al. 2011; Zhao et al. 2003). One of the studies highlighted their epigenetic role in adult neurogenesis through MeCP2, which is a DNA methyl-CpG–binding protein that is essential for normal function of nerve cells and is involved in several diseases (Guy et al. 2011) including Rett Syndrome (Zoghbi et al. 1999). MeCP2 is also involved in the regulation of miR-137 expression in the adult brain by altering the epigenetic status in the chromatin surrounding miR-137, which results in premature miR-137 expression (Szulwach et al. 2010). miR-137 is an abundant miRNA in the adult brain and its altered expression induces differentiation and inhibits proliferation of embryonic neuronal stem cell (Kozaki et al. 2008; Mahmoudi & Cairns 2017). This indicates that cross talk between miRNA and epigenetics plays a role in the maintenance of adult neurogenesis.

miRNAs are a newly emerged group of small non-coding RNAs that have been found to be involved in post transcriptional regulation of gene expression (Lee et al. 1993). Analysis studies of miRNAs identified several that are highly expressed in the CNS including miR-124, miR-132, miR-134, miR-137, miR-218 (Reviewed in (Cao et al. 2016). The role of miRNAs in adult neurogenesis has been explored in recent studies. For example, the activity of miR-124 has been associated with neuronal differentiation during adult SVZ neurogenesis. miR-124 functions through the activation of Sox9 to regulate the transition from the transit amplifying cell (type C cells) to the neuroblast stage (type A cells) (Cheng et al. 2009). Additionally, miR-17-92 cluster was found to regulate adult hippocampal neurogenesis and is

involved in the development of anxiety-like behavior through the regulation of glucocorticoid inducible protein kinase-1 *SKG1* gene (Jin et al. 2016). Moreover, miR-19 activity is mediated by suppressing Rap guanine nucleotide exchange factor 2 (Rapgef2) to regulate the positioning and migration of NPCs in hippocampal adult neurogenesis (Han et al. 2016). Factors that are involved in the regulation of adult hippocampal neurogenesis are summarised in (Figure 1-4).

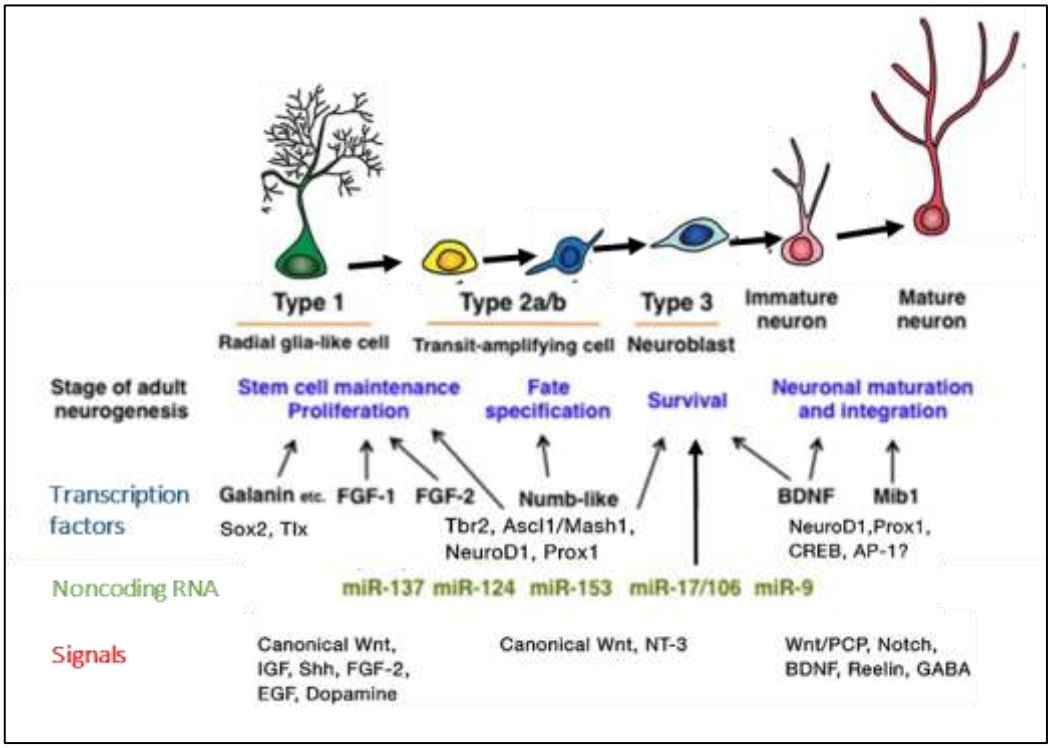


Figure 1-5: Schematic diagram showing factors involve in the regulation of adult hippocampal neurogenesis.

Adult hippocampal neurogenesis has 4 main stages: (1) stem cell maintenance and proliferation, (2) fate specification, (3) survival, and (4) neuronal maturation and integration into the existing neural circuits. Type of cells in the figure: [1] Radial glia-like cells (type 1) in the SGZ, [2] proliferating precursor cells, transit-amplifying cells (type 2a, 2b), [3] Neuroblasts (type 3), [4] differentiation into immature neurons, followed by their maturation. Factors regulate neurogenesis including, transcription factors, non-coding RNAs and signalling pathways. Noncoding RNAs regulate adult hippocampal neurogenesis by mediating their target genes and related pathways. The figure was adapted from (Murao et al. 2016).

Identification of possible interactions between microRNAs and key players in neurogenesis can reveal novel regulatory molecular mechanisms. This in turn may help to answer questions relating to aspects such as neural development, plasticity as well as possibly having therapeutic potentials in several diseases such as neurodegenerative diseases, traumatic brain injury (TBI) and spinal cord injury (SCI) (Sun & Shi 2014; Zhao et al. 2014; Redell et al. 2010).

1.6.2 Extrinsic factors

Neurogenesis can be regulated by extrinsic factors, including: ageing, stress, environment enrichment (EE) and diet. Those factors can have both positive and negative effects on the different stages of adult neurogenesis.

An EE is an environment that provides exposure to social, sensory and motor stimulation. Previous work has shown that mice spending time in an EE had more proliferative cells, more NPCs differentiating into neurons in comparison to control and more surviving cells (Kempermann et al. 1997). Vascular endothelial growth factor (VEGF) is one important growth factor that is upregulated by EE, as inhibition of VEGF inhibits EE-induced neurogenesis (Cao et al. 2004). BDNF is another growth factor regulated by EE which leads to the enhancement of hippocampal adult neurogenesis (Rakhit et al. 2005). Additionally, it has been found that BDNF is associated with memory consolidation, as the inhibition of BDNF local protein expression in the dorsal hippocampus resulted in impaired long term memory consolidation (Lee et al. 2004).

Studies have also linked learning with neurogenesis. Learning behaviours (or tasks) have been found to improve the survival of newly generated neurons within the hippocampus, in addition to improving maturation of newly generated neurons (Dupret et al. 2007). Rats that underwent Morris water maze (MWM) testing, a hippocampal dependent spatial memory paradigm, exhibited increased axonal density and more complex arborisation in the CA3 region (Lemaire et al. 2012).

Diet is another extrinsic factor that has a tremendous effect on hippocampal neurogenesis and cognitive functions. Experiments on rodents have shown that food lacking essential vitamins and minerals negatively affects adult neurogenesis, while food rich with fatty acids has been shown to improve cognitive performance and increase adult neurogenesis (Stangl & Thuret 2009). Despite the importance of these studies, it is still challenging to address how each component is absorbed and metabolised, and which specific factor acts to affect neurogenesis. Along with the difficulty of performing *in vitro* studies to support applied hypotheses, it is difficult to investigate specific dietary factors and their effects on neuronal survival or proliferation. However, several studies have been conducted on elderly humans to address the effect of flavonoid, include berries, tea and chocolate consumption on neurogenesis, as the neurogenesis rate declines with age and can result in reduced cognitive functions. This study found improved cognitive functions during the 10 year period (Letenneur et al. 2007). Nevertheless, in humans there are many variables that cannot be controlled, which makes it difficult to determine the effect of each factor or to find the most influential one among the others, and to address which of the cognitive functions is affected the most by dietary intake.

One of the factors that negatively regulates neurogenesis is aging. Aging is associated with decline in NPC proliferation, survival and neural differentiation. BrdU-labelled proliferating cells are reduced significantly in aged mice (Shoji et al. 2016; Dennis et al. 2016). Consistently, BrdU colocalisation with NeuN (neuronal marker) was reduced with age, indicating a decline in neural differentiation with age (Kuhn et al. 1996b; Dennis et al. 2016). Interestingly, when older mice (10 months old) were exposed to EE for another 10 months, they exhibited a significant increase in adult new born neurons compared to control and performed better in MWM test (Kempermann et al. 2002). This emphasises the importance of EE in improving neurogenesis and reducing the negative effects associated with aging.

Stress is another negative regulating factor for neurogenesis and this is associated with the release of glucocorticoids into the bloodstream. It has been indicated that corticosteroid treatment led to reduced neurogenesis in the DG, while its removal resulted in increased neurogenesis indicating that stress is a negative regulator of adult neurogenesis (Cameron & Gould 1994). Generally, animals exposed to stress such as predator sounds, isolation in an empty cage, placement in a cage with water on the bottom, inversion of the light/dark cycle, and cage-switching all exhibited reduced proliferation, survival and neural differentiation in the DG (Mineur et al. 2007). Studies have investigated the effect of both acute and chronic stress on adult neurogenesis using different stress stimuli such as, physical, social and odour stressors, and found that in most cases exposure to chronic stressors resulted in a reduction in proliferation and growth of newborn neurons (Mineur et al. 2007),

however, several reports associated acute stress with increased neurogenesis (Kirby et al. 2013).

1.7 The use of mice as an animal model to investigate neurogenesis

The mammalian brain retains the ability to generate new neurons during the adult stage. The rate of generation of new neurons seems to be affected by several factors including age, psychiatric disorders and neurodegenerative diseases. Human brain banks provide brain tissue which has been fixed or fresh frozen to enable the experimental use of the tissue to address the influencing mechanisms involved. Although there are marked differences between adult neurogenesis in humans and rodents including the rate, location of neurogenic areas and factors involved, the limitations of using human brains in research have led to continued use of rodent models to study neurogenesis, particularly with the advantage of using tools such as viral labelling and transgenic mice to manipulate gene function (Ernst & Frisén 2015). Moreover, rodent models present useful experimental paradigms to better understand the molecular mechanisms regulating NPC production and survival, as assessed by genetic manipulation, immunohistochemistry, electrophysiology and functional behaviour testing. A general approach to start with is to use green fluorescent protein (GFP) to label a certain population of neurons, which can be combined with cell specific markers to visualise cells in live animals or in acute brain slices, with the additional benefit of performing directed electrophysiological studies. Knock out transgenic mice are useful tools for analysing specific gene or non-coding RNA function. This process is highly reliable on the availability of the right promoter elements, as transgenic expression can be directed to a specific population in the

nervous system, like neurons or astrocytes, or have a more global expression in the whole body by selecting a universal promoter such as the CAG promoter, which is used to introduce high expression levels vectors (Miyazaki et al. 1989). Examples of available transgenic mice to study neural stem and progenitor cells in adult neurogenesis are nestin-based transgenic mice models (Yamaguchi et al. 2000), doublecortin-based transgenic mouse line and pro-opiomelanocortin-alpha POMC-GFP expressing neurons, which enable the identification of post mitotic granule cells (Couillard-Despres et al. 2006; Overstreet et al. 2004).

1.7.1 Detection methods for mouse adult neurogenesis

Detection of adult neurogenesis *in vivo* has advanced greatly during the last decade due to the improved technology for detecting and quantifying adult newborn neurons in live animals, such as magnetic resonance imaging (MRI), and the use of *in vivo* microscopy including deeply penetrating UV illumination with multiphoton microscopy and endoscopic confocal microscopy. These advances in technology are particularly useful when coupled with transgenic mice expressing a specific phenotype. Another advanced method is measurement of the concentration of the radioactive carbon-14 isotope (^{14}C) in genomic DNA in post-mortem brains (Spalding et al. 2013). Despite these advances in the latest technology, the most popular method for investigating adult neurogenesis is the traditional histological method using thymidine analogs such as bromodeoxyuridine (BrdU) and ethynyldeoxyuridine (EdU) that are incorporated into dividing cells and staining with neuronal specific markers such as DCX and NeuN, to identify proliferating cells within the brain. Additionally, using retrovirus cell labelling has enable the specific labelling of

dividing cells and their progeny, which can be used to study the molecular mechanism involved between different cells during adult neurogenesis. As retrovirus integration is dependent on the M phase in the cell cycle, this means that only dividing cells are going to have the expression of the marker gene such as alkaline phosphatase (AP) and GFP.

1.7.2 Markers for adult NPCs and neurogenesis

The CNS is derived exclusively from the neuroectoderm, a sheet of primitive neural plate during development. The neuroectoderm eventually forms the neural tube, which gives rise to the brain and spinal cord. A population of both neural stem and progenitor cells are essential to the development of the CNS. Neural stem cells are defined as cells that can undergo self-renewal and divide to generate multipotent progenitor daughter cells that have the ability to differentiate into all three neural lineages – neurons, astrocytes and oligodendrocytes (Temple 2001).

To study the early events in development such as self-renewal and lineage commitment, suitable markers are needed. Markers expressed during this phase can be a good indicator of ongoing neurogenic activity. These markers ideally need to be expressed only in the population of cells committed to specific phases of the neural lineage, without being expressed in other brain cell types, or in other conditions like injury or regeneration. Although the use of markers enables the identification of areas with new neurons, it has some limitations, as there is no neural stem cell marker that is definitive for neural stem cells. Nestin is the most widely used marker for neural stem cells, but it can be detected in vascular structures. Therefore, a combination of other markers is needed for more precise identification, for example: glial fibrillary

acidic protein (GFAP), which is a marker for astrocyte and radial glial cells, and sex determining region Y (Sox-2), which is another neuronal stem cell marker that can be combined with nestin (Alvarez-Buylla & Garcia-Verdugo 2002). One widely used immature neuronal marker is doublecortin (DCX) which is a microtubule associated protein and a migration marker (Brown et al. 2003). Another marker is βIII-tubulin (also known as Tuj1), which is expressed in early committed neurons and in mature neurons. When progenitor neuronal cells reach maturity, they expressed neuronal markers such as NeuN as well as calcium-binding proteins such as Calbindin, calcium-binding protein G (S100G) and Calretinin (CR), which are proteins that participate in the calcium signalling pathway. The use of specific markers to identify cells at various differentiation stages is summarized in Figure 1.5. These specific markers in combination with BrdU birth dating, can be used for the identification and quantification of newly born neurons (Kempermann et al. 2003).

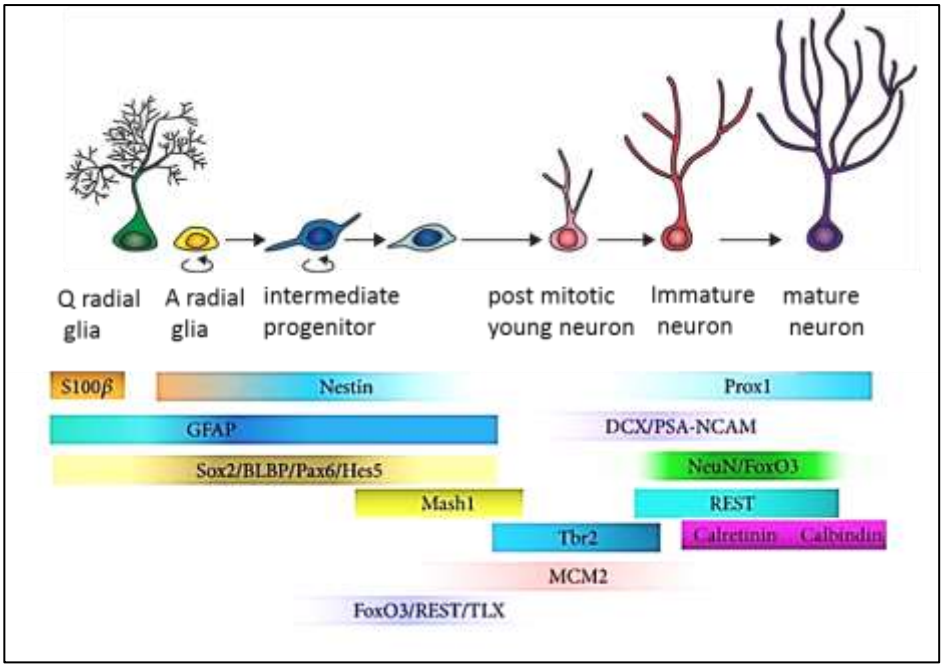


Figure 1-6: Markers for adult NPC and neurogenesis.

The figure illustrates specific markers which are used to detect for specific cell types in different time-courses. Figure adapted from J. Zhang & Jiao (2015).

1.8 MicroRNA

In 1993, lin-4 was described as the first short non-coding RNA involved in the regulation of development timing in the nematode *C. elegans* (Lee et al. 1993). During development this worm undergoes a series of precisely timed post-embryonic divisions and continues development through four larval stages (L1–L4) until the worm reaches adulthood. Lin-4 is one of the regulatory miRNAs involved in the developmental patterns of all larval stages. It has been found that loss of lin-4 in *C. elegans* caused developmental problems including loss of the ability to lay eggs and preserved earlier developmental features at later larval stages (Chalfie et al. 1981). Additionally, it has been found that the loss of *Lin-14* gene function

resulted in the reverse of the effect observed with lin-4 loss, suggesting that lin-4 negatively regulates *Lin-14* gene (Ferguson et al. 1987). The mechanism of regulation was interesting, as an isolated *lin-4* gene did not produce mRNA to encode the gene; instead it produced a non-coding RNA that had a complementary sequence to the *Lin-14* mRNA 3' end untranslated (3'UTR) region, which follows the translation termination codon of mRNA (Bartel 2004). It was suggested that this complementarity inhibited the translation of the *lin-14* mRNA into the Lin-14 protein and functioned as a post transcriptional regulatory mechanism. However, the discovery of small RNAs was not being taken seriously until the discovery of RNAi and let-7 microRNA, when it was reported that let-7 was able to inhibit lin-14, lin-28, lin-41, lin-42 and daf-12 expression during early developmental stages in *C. elegans* (Reinhart et al. 2000). The conserved expression of let-7 RNA suggested that it may be involved in the regulation of developmental timing in animals and humans in a manner similar to lin-4 function (Roush & Slack 2008). The let-7 family consists of nine different miRNAs, with the same seed sequence including and includes let-7, miR-84, miR-48 and miR-241, which are expressed in *C. elegans,* and are involved in the sequential regulation of genes involved in cell fate decision making during larval development (Thummel 2001). To date an increasing number of miRNAs have been discovered, with each one of them potentially regulating up to hundreds of genes. miRNA expression is important to control gene expression under normal conditions, but it can also become dysregulated in diseases with implications for cancer (Calin et al. 2002), heart diseases (Zhao et al. 2007), kidney diseases (Phua et al. 2015) and diseases of the nervous system (Maes et al. 2009).

1.8.1 Biogenesis of miRNAs

MicroRNAs (miRNAs) are 20-24 nucleotide long single-stranded RNA molecules that regulate protein expression at a post transcriptional level (Bartel, 2004; Lee et al., 1993). They are produced from either their own genes or from introns, and arise from primary RNA transcripts (pri-miRNA) that can be thousands of base pairs in length (Han et al. 2004). Biogenesis of miRNAs is illustrated in Figure 1.6, and it begins with the transcription of the miRNA gene by RNA polymerase II to produce primary miRNAs (pri-miRNAs) (Han et al. 2004). Pri-miRNAs form significant and functional secondary structures that contain a stem-loop structure (Han et al. 2004; Zeng et al. 2003). The stem-loop structure contains an RNA sequence of approximately 24 nucleotides that has a complementary nucleotide sequence a few nucleotides away, which allows the complementary regions to base pair and form the stem-loop structure. This structure is recognised by the enzyme Drosha, an RNAse III endonuclease (Basyuk et al. 2003; Han et al. 2004). Binding of Drosha results in RNA excision to form the "Microprocessor" complex, liberating the precursor-miRNA (pre-miRNA) from the pri-miRNA. The pre-miRNA hairpin is exported from the nucleus into the cytoplasm by exportin-5 (Yi et al. 2003; Lund et al. 2004). In the cytoplasm Dicer cleaves pre-miRNA to yield an imperfect miRNA:miRNA* duplex about 22 nucleotides in length (Lau et al. 2001; Chendrimada et al. 2005). Although both strands of the duplex may act as a functional miRNA, only one strand is incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact (Long & Lahiri 2012).

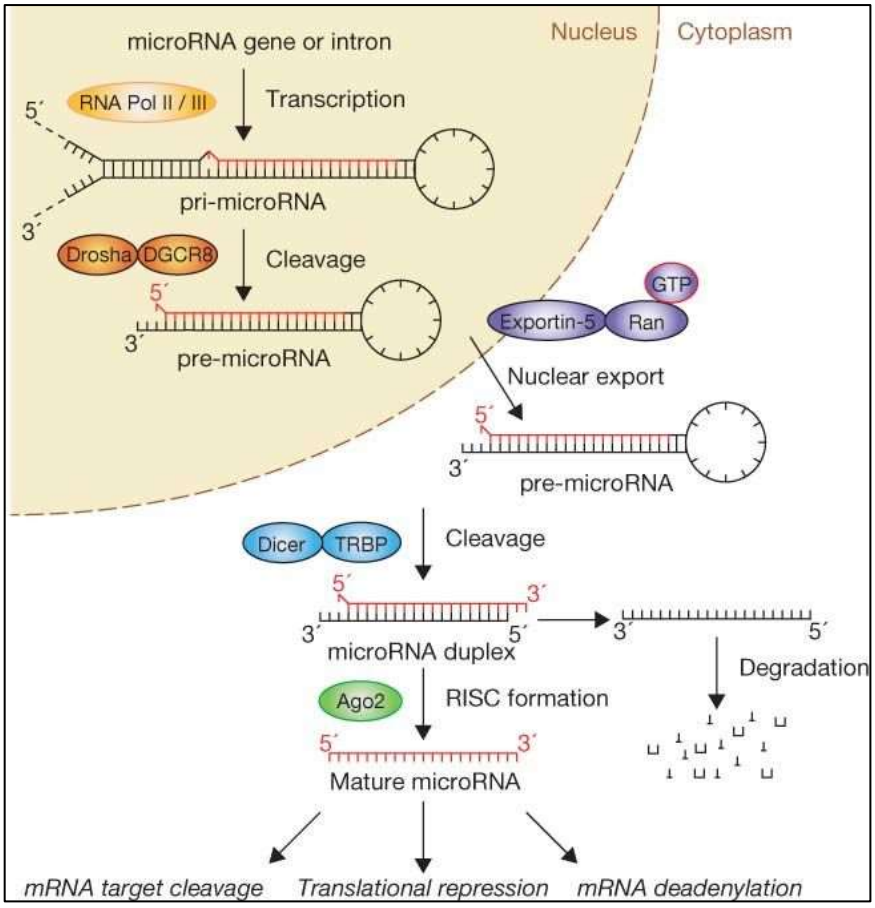


Figure 1-7: MiRNA biogenesis.

miRNAs are small non-coding RNAs about 18-22 nt long, that are derived from either an intrinsic or extrinsic region. They are mostly transcribed by the RNA polymerase II into long primary loop transcripts (pri-miRNA), which are cleaved by the microprocess complex DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha. The precursor miRNA (60 nt) is then exported to the cytoplasm by Exportin 5 transporter, where it is further processed by Dicer and TRBP (transactivation-response RNA-binding protein) to form the mature form of miRNA duplex. The most thermodynamically stable strand of the miRNA duplex is selected by the Argonaut Ago 1-4 family as the guide strand, while the other is degraded or can be used as another mature strand. miRNA is assembled into a miRNA induced silencing complex (RISC) to be guided to the target mRNAs. miRNA binds to the (3' UTR) region of the target mRNAs, and either degrades or silences them depending on the degree of similarity between the seed region (2' and 8') in the 5' end. Figure taken from (Winter et al. 2009).

The RISC contains the Argonaute family of proteins and is responsible for miRNA targeting and binding to messenger RNAs (Hammond et al. 2000; Martinez et al. 2002; Peters & Meister 2007). MiRNAs target mRNA species that contain the complementary sequences to the miRNA seed sequence. The level of binding between the 20-24 nucleotide miRNA and mRNA is affected by their level of complementarity. In particular, the 5' end nucleotides two to eight of the miRNA sequence, termed the seed sequence, which plays a critical role in miRNA: mRNA binding (Lewis et al. 2003). These binding sites are conserved and found in the 3'UTR of mRNA (Bartel, 2004). Degradation of the target mRNA will occur in cases of 100% complementarity between miRNA and mRNA, which happens in the case of plants; however, in cases of lower complementarity translation of the target mRNA is repressed. The imperfect complementarity between miRNAs and their target mRNAs can result in a mRNA that is regulated by a large number of miRNAs (Wu et al. 2010; Peter 2010). For example p21 had been found to be targeted by 28 miRNAs, and this can lead to the enhancement of proliferation enhancement and alterations in cell cycle progression (Lim et al. 2005; Wu et al. 2010). Therefore the fact that one miRNA can bind to more than one mRNA and conversely, a given mRNA can be bound by multiple miRNAs means that some miRNAs can modulate a family of genes that are related functionally, enhancing the miRNA effect (Lee et al. 2004; Oliveto et al. 2017).

It has been reported that miR-21 has two mature isoforms, miR-21-3p and miR-21-5p (Jiao et al. 2017). It has long been held that each arm of the pre-miRNA produces one consequential mature miRNA. However, subsequently isoforms (isomiRs)

discovered to be arised from the same arm of miRNA which demonstrated different 5′ and/or 3′ termini. Both miR-21-3p and miR-21-5p have implications in cancer and other diseases, despite miR-21 -5p is more conserved and has more significant disease potentiality than miR-21-3p (Telonis et al. 2015).

1.9 Role of miRNAs in neurogenesis

Recent work has been carried out to investigate the link between miRNAs and neurogenesis. miRNAs play indispensable roles in brain development, neurogenesis and in modulating transcriptional networks (Kosik & Krichevsky 2005; Mehler & Mattick 2007). Given the universal expression of miRNAs, global depletion of miRNAs was achieved by targeting major enzymes involved in their production such as the complete Dicer knock-out mouse, which displayed abnormal brain development with overproduction of cortical neurons (Liu et al. 2004). In addition, conditional ablation of Dicer in the CNS resulted in a smaller cortex in the knock-out mice (Tonelli et al. 2008). The loss of another important enzyme called Drosha in forebrain neural progenitors resulted in the loss of stem cell characteristics and early differentiation (Knuckles et al. 2012).

It has been demonstrated that specific miRNAs are involved in the regulation of neurogenesis. Therefore, research has focused on investigating miRNA targets in a context-dependent way. miR-124 is one of the abundant microRNAs in CNS. Expression levels for miR-124 increase during brain development, and upon neuronal differentiation, reach maximal levels in mature neurons (Lagos-Quintana et al. 2002). miR-124 has been shown to regulate NPC differentiation in the SVZ by targeting the factor Sox9 (Cheng et al. 2009). Overexpression of miR-124 has been found to

promote neuronal differentiation, whereas knockdown of miR-124 preserves NPC precursor identity. In particular, the miR-124-mediated feedback regulatory loop mechanism plays important roles in modulating neural cell fate and neurogenesis (Cheng et al. 2009).

MiR-137 expression is epigenetically controlled by methyl CpG binding protein 2 (*MeCP2)* and *Sox2*, and its overexpression and deletion corresponds to NPC proliferation and differentiation respectively. miR-137 targets the H3K27me3 methyl transferase Ezh2, which may lead to the modulation of neurogenesis-related genes (Szulwach et al. 2010). Moreover, miR-137 forms a regulatory loop with *TLX* and *LSD1*, which is a direct target of miR-137. Overexpression of miR-137 in mouse embryonic brains leads to premature differentiation. Meanwhile, miR-137 is negatively regulated by the transcription factor *TLX*, showing another example of TLX-mediated neurogenesis (Sun et al. 2011).

Therefore, microRNAs' involvement with different epigenetic mechanisms ensures the precise control of neurogenesis and correct neuronal functions. More miRNAs are predicted to have critical roles in adult neurogenesis due to the development of deep sequencing techniques. Recently, miR-19 has been shown to regulate the migration and maturation of newborn neurons in the granule cell layer of the DG by suppressing Rap guanine nucleotide exchange factor 2 *(Rapgef 2)* (Han et al. 2016). Some other known miRNAs associated with hippocampal adult neurogenesis regulation are illustrated in (Table 1-1).

miRNAs	Target	miRNA function	Origin of Identification	Reference
miR-184	*Numbl*	Inhibits differentiation, promotes proliferation, maintenance of the neurogenic stem cell pool	Mouse NPCs	(Liu et al. 2010)
miR-34a	*Numbl*	Inhibits differentiation, promotes proliferation, maintenance of the neurogenic stem cell pool	Mouse NPCs from embryonic cortex	(Fineberg et al. 2012)
	BCL2	Promotes apoptosis	Mouse cortex/SH-SY5Y cell line	(X. Wang et al. 2009)
	Cdk-4, Cyclin D2	Inhibits cell cycle progression	Primary keratinocytes	(Antonini et al. 2010)
	Synaptotagmin1, Syntaxin-1A	Inhibits synaptic development	Mouse ES cells	(Agostini et al. 2011)
miR-106b/miR-25 Cluster	*IGF-signalling*	Promotes NSPC proliferation, neuronal differentiation	Mouse primary NPC culture	(Brett et al. 2011)
miR-124	*Sox9*	Promotes neuronal differentiation	Mouse NPCs	(Cheng et al. 2009)
	STAT3	Promotes neuronal differentiation	Mouse ESCs	(Higuchi et al. 2016)
	BCL2L13	Inhibits apoptosis	Mouse DG	(Schouten et al. 2015)
	Lhx2	Promotes neurite outgrowth	Mouse hippocampus	(Sanuki et al. 2011)
	Rap2a	Promotes dendritic branching	Mouse NPCs	(Xue et al. 2016)
miR-137	*BCL2L13*	Inhibits apoptosis	Mouse DG	(Schouten et al. 2015)
	Mib-1	Inhibits dendritic growth and spine formation	Mouse hippocampus & mouse primary neuronal culture	(Smrt et al. 2010)
	LSD1	Promotes proliferation	Embryonic NPCs	(G. Sun et al. 2011)
	Ezh2	Inhibits differentiation	Adult NPCs	(Keith E Szulwach et al. 2010)
miR-9	*TLX*	Promotes differentiation, inhibits differentiation	Mouse hippocampus	(Zhao et al. 2009)
	REST	Promotes neuronal differentiation	NT2 cell line	(Packer et al. 2008)
	Rap2a	Promotes dendritic branching	Mouse NCSs	(Xue et al. 2016)

miRNAs	Target	miRNA function	Origin of Identification	Reference
	Stathmin	Inhibits migration	Human embryonic NPCs	(Delaloy et al. 2010)
Let-7b	*TLX*	Inhibits proliferation, promotes differentiation	Mouse NPCs & embryonic mouse brain	(Zhao et al. 2010)
	Cyclin D1	Inhibits cell cycle progression	Embryonic mouse brain	(Zhao et al. 2010)
miR-125b	*Nestin*	Promotes neuronal differentiation	Rat NSPCs	(Cui et al. 2012)
miR-145	*Sox2*	Promotes neuronal differentiation	Mouse NPC	(Morgado et al. 2016)
miR-26b	*Ctdsp2*	Promotes neuronal differentiation	Zebrafish & P19 cells	(Dill et al. 2012)
miR-19	*Rapgef2*	Stimulates migration	Mouse hippocampus & mouse NPCs	(Han, Joon Kim, et al. 2016)
miR-379/miR410 cluster	*N-Cadherin*	Induces migration	Mouse embryonic cortex	(Rago et al. 2014)
miR-134	*Limk1*	Inhibits spinogenesis	Rat hippocampus	(Schratt et al. 2006)
	DCX	Inhibits NPC migration	Primary NPCs, primary neurons, mouse embryonic cortex	(Gaughwin et al. 2011)
miR-138	*APT1*	Inhibits spinogenesis	Mouse hippocampus	(Siegel et al. 2009)
miR-17/miR92 cluster	*PTEN*	Induces axonal outgrowth	Rat primary cortical neurons	(Jin et al. 2016a)
miR-132	*P250GAP*	Promotes spinogenesis	Mouse hippocampus	(Impey et al. 2010)

Table 1-1: miRNAs involved in the regulation of hippocampal adult neurogenesis.

Protein targets, role and origin of identification are illustrated. The table was taken from (Bielefeld et al. 2017).

1.9.1 MiRNA-21

The microRNA miR-21 is conserved in mammals and is encoded by a single gene. In humans, the pri-miR-21 sequence maps to chromosome 17q23.2 and in mice, it is found on chromosome 11 (Figure 1-7). In both species, the miR-21 gene is found in the 3'UTR of the gene encoding for vacuole membrane protein 1 *(VMP1)*, which in itself is not a target of miR-21, and regulated independently from that of *VMP1* (Dusetti et al. 2002; Fujita et al. 2008; Ozsolak et al. 2008; Krichevsky & Gabriely 2009). Additionally, deep sequencing has identified a consensus sequence within the miR-21 promoter region that contains several conserved enhancer elements (Fujita et al. 2008; Muppala et al. 2013; Du et al. 2009; Han et al. 2012; Yang et al. 2013). Transcription factors that regulate miR-21 expression include: AP-1, NFIB, β-catenin, p53, CD24 and STAT3 (Löffler et al. 2007).

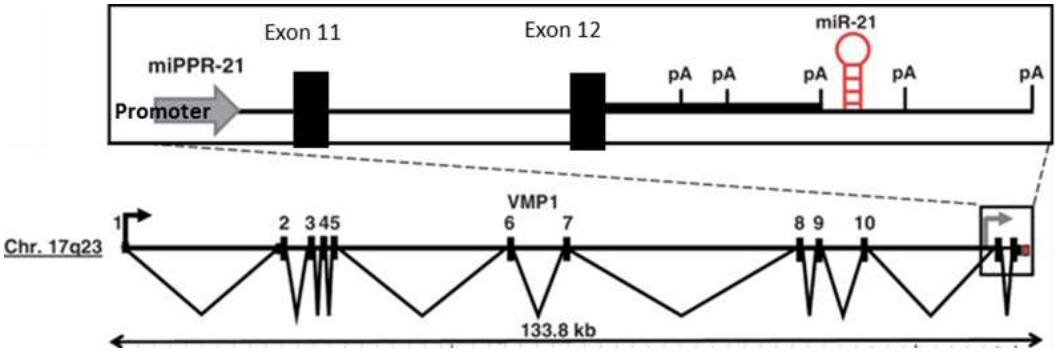


Figure 1-8: Structure of miR-21 and VMP1.

miR-21 is located on chromosome 17q23 and despite its close proximity downstream of the *VMP1* coding gene, it is regulated by different promoters. *VMP1* exon numbers are exons 11 and 12 of *VMP1*. A grey arrow is situated at the miR-21 promoter, miPPR-21. The red hairpin indicates the location of the pre-miR-21 hairpin. The black arrow represents the transcription start site of *VMP1*.Figure adopted from Ribas et al. (2012).

miR-21 was among the first miRNAs to have the attention of researchers, because of its strong link to cancer. miR-21 is overexpressed in a large number of cancer types, including glioma the most malignant brain tumour of glial origin, kidney, lung, breast, stomach and leukemia (Krichevsky & Gabriely 2009). Additionally, miR-21 is up-regulated in cardiac hypertrophy and can be upregulated in a variety of other human cell lines that usually express low or undetectable levels of miR-21, such as mouse embryonic stem cells, neuroblastoma human SHSY5Y, and myeloid line HL-60 (reviewed in (Krichevsky & Gabriely 2009)). Comparison of expression levels between normal and diseased tissues with pathological cell growth or even cell stress showed that miR-21 is highly expressed in most of the cancerous tissues and cell lines by at least two fold compared to a very moderate expression level in normal tissues (Buscaglia & Li 2011). MiR-21 is detected at higher levels in the neonatal and the early embryogenesis stages, with expression becoming gradually reduced until it reaches a very low or undetectable level as development proceeds (Põlajeva et al. 2012). This regulation of miR-21 expression levels has been linked to the activity of Ras pathway, which is mediated by STAT3 activity to regulate cell growth, differentiation and survival (Ou et al. 2014). This form of regulation during early development is consistent with suggested function of miR-21, as high expression levels promote proliferation, and low expression levels promote cell fate differentiation during the later developmental stages.

There is considerably more literature on miR-21 functions related to the regulation of apoptosis, survival and proliferation, as it has been found to be linked to the progression of many cancer types including glioma, than the relatively recent

identified miR-21 role in tissue repair after traumatic brain injuries. This will be further discussed in the following sections.

1.9.2 MiR-21 expression in the brain

Using quantitative PCR, miR-21 has been detected in multiple regions of the normal brain (Ge et al. 2014; Redell et al. 2011). Additionally, detection by *in situ* hybridization (ISH), which enable the visualization of miRNAs signal in the selected tissue, has reported miR-21 expression in the brain (Bhalala et al. 2012; Buller et al. 2010; Põlajeva et al. 2012; Ge et al. 2015). miR-21 is highly expressed during early brain development in both the hippocampus and the cortex and its expression is associated with the stem cell marker SOX2 (Põlajeva et al. 2012). In the adult brain, another study detected low levels of miR-21 expression (Harrison et al. 2016a; Liu et al. 2016). In addition, miR-21 has been detected in the non-diseased CNS as well as in the neurogenic cell lines HT22 and N2 (Lagos-Quintana et al. 2001; Krichevsky et al. 2003; Landgraf et al. 2007). Co-localization of miR-21 with cell type specific markers showed expression in neurons, astrocytes and microglia (Ge et al. 2015; Simeoli et al. 2017). It is important to note that the abundance of miRNAs does not always reflect less significant functions, as some miRNAs with low expression levels still have the potential to impact on crucial functions. For example, BOTH miR-206, which has been implicated in colon cancer and regulates *KLF4* expression and miR-29a, which is involved in hepatic disease, play significant roles in disease despite low expression levels (Zhang et al. 2010; Wang & Gu 2012). Although low abundance miRNAs might not reflect a measurable biological phenotype, this should not completely exclude their possible importance. Thus, low expression levels of miR-21

in a normal brain may still represent an important molecular mechanism for regulating hemostasis.

Importantly, miR-21 levels in the brain and the spinal cord is significantly increased after injury. This increased expression was linked to elevated expression of miR-21 has also been observed in HIV-associated neurocognitive disorders and found to contribute to neuronal dysfunction (Yelamanchili et al. 2010). Additionally, regenerative effects of miR-21 were observed in models of neurodegeneration, axotomy and stroke (Buller et al. 2010b; Strickland et al. 2011; Montalban et al. 2014).

1.9.3 MiR-21 in cancer

Although cells express over 1000 different miRNAs, few are as much studied as miR-21. MiR-21 was first identified because of its high expression levels in glioblastoma multiform tumors isolated from various patients (Chan et al. 2005; Ciafrè et al. 2005). Large scale miRNA profiling studies of over 500 cancers consisting of 6 different cancer types compared to 170 normal controls identified miR-21 as the only miRNA up-regulated in all types of cancers analysed (Volinia et al. 2006). Subsequently, miR-21 has been found in almost every type of cancer, including breast, lung, pancreas, stomach, prostate hepatocellular carcinoma, leukemia and other types of cancer (Krichevsky & Gabriely 2009).

Overexpression of miRNA-21 increases lung tumour formation, while its absence slows tumour progression (Hatley et al. 2010). Moreover, overexpression in hematopoietic stem cells can induce leukemic transformation and when miR-21

levels revert to baseline, the leukemia regresses (Medina et al. 2010). Because of its involvement with many different types of cancer, miR-21 is described as an oncogene or an onco-miR. MiR-21 is closely linked with cancer because many of its targets, such as phosphatase and tensin homolog *(PTEN)* and programmed cell death 4 *(PDCD4)*, sprouty homolog 1 *(SPRY1)*, Protein sprouty homolog 2 *(SPRY2)* and BTG family member 2 *(Btg2)*, are negative regulators of cancer propagation (Krichevsky & Gabriely 2009). Additionally, miR-21 can inhibit apoptosis through B-cell translocation gene 2 (*Bcl-2)* (Ge et al. 2014), pro-apoptotic FAS ligand (*FasL*) (Zhang et al. 2012), inhibitor of metalloproteinases 3 (*TIMP3*), and Apoptotic protease activating factor 1 (*Apaf1)* (Hatley et al. 2010). Moreover, studies have suggested that miR-21 levels can determine whether a cell differentiates (miR-21 levels are low to moderate) or becomes oncogenic (miR-21 levels are very high) (Fujita et al. 2008). Thus, miR-21 can be used as a universal biomarker for cancers.

1.9.4 MiR-21 in traumatic brain injury

Traumatic brain injury (TBI) is any damage to the brain resulting from external mechanical force, which may cause disability or death depending on its severity. TBI has two stages: primary and secondary brain damage. Primary brain damage is caused by the immediate impact of the injury and leads to cell necrosis. Secondary brain damage occurs in the hours and days after primary brain damage and is characterised by inflammation, activation of apoptotic pathway, activation of necrotic pathway and lastly apoptosis (McAllister 2011). Studies have shown that miRNAs can be used as biomarkers for altered physical conditions in the brain following TBI, including miR-16, miR-92a, and miR-765, which all show increased expression related to the injury

(Redell et al. 2010; Ge et al. 2015). Interestingly, several studies have shown increased expression of miR-21 in the injured area after TBI (Ge et al. 2015; Redell et al. 2011). Increased miR-21 expression has been shown to correlate with reduced lesion sizes and improved cognitive performance after brain injury (Ge et al. 2014). This increased expression of miR-21 via miR-21 agomir has been linked to activation of Angiopoietin-1 (Ang-1) and Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-2), which are genes that promote the expression of tight junctions and can therefore reduce lesion size. However, inhibition of miR-21 via miR-21 antagomir had the opposite effects (Ge et al. 2014). MiR-21 also caused a decrease in apoptotic activity through the activation of AKT signalling pathway and upregulation of the tumor suppressor protein PTEN. MiR-21 has been shown to increase Bcl-2 protein expression and inhibited Bcl-2 associated X protein Bax, Caspase-3 and Caspase-9 expression in the brain, thus suggesting that miR-21 inhibits cellular apoptosis (Ge et al. 2015). In another study, miR-21 brain expression levels were compared in adults and aged mice after injury (Sandhir et al. 2014). A reduced level of miR-21 was detected in aged mice and this was correlated to increased expression of the target genes *PDCD4*, *RECK*, *TIMP3* and *PTEN*, resulting in increased pathology and poor prognosis after TBI, indicating that reduced expression levels of miR-21 in aged mice is linked to the poor functional outcome (Sandhir et al. 2014).

1.10 Hypothesis and aims of the study

The unique neurogenic niches in the adult mammalian brain, in which new neurons continue to be generated throughout life, has opened the door to a new area of brain research. Generation of these precursor neurons within these neurogenic niches is affected by extrinsic and intrinsic regulators, and further investigation of underlying molecular mechanisms will prove crucial to understanding the process. Also, it is important to understand how the interactions between extrinsic and intrinsic regulators control the physiological and pathological nature of the neurogenic niches to maintain homeostasis, as the failure to regulate neurogenesis can result in many neuropsychiatric and neurodegenerative diseases. Understanding the mechanism that controls neurogenesis may help to overcome the progression of pathogenic symptoms associated with these diseases.

The microRNA miR-21 is highly expressed in the developing brain. In addition, miR-21 has been demonstrated to have a positive effect on neurogenesis after SCI and TBI, as increased levels of miR-21 have been shown to alleviated brain edema and decreased lesion volume. Additionally, recent research has illustrated that certain miRNA knockouts and overexpression models such as miR17-92 and miR-19 can alter adult neurogenesis, suggesting a possible therapeutic use for miRNAs in neuropsychiatric or neurodegenerative diseases (Han et al. 2016; Jin et al. 2016).

Our hypothesis is that overexpression of miR-21 will have beneficial effects on adult neurogenesis under normal conditions, by elevating the number of adult born cells in neurogenic regions via increased proliferation or/and inhibition of apoptosis, while its loss will result in the opposite effects. Therefore, the overall aim of this research

is to investigate the role of miR-21 in adult neurogenesis in normal adult brain; this dissertation will present data that demonstrate:

1. expression patterns of miR-21 in the central nervous system;
2. Functional effects of miR-21 overexpression or knockdown on hippocampal neurogenesis;
3. Assessment of effects of miR-21 overexpression or knockdown on subventricular zone (SVZ) neurogenesis;
4. potential molecular mechanisms that underly the biological effects of miR-21

Chapter 2 Methods

2.1 Animals

All mouse experiments were approved by the University of Bristol Ethical Review Group and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Animals were maintained on a 12-h/12-h light/dark cycle in a controlled temperature and humidity room with access to food and water. miR-21KO and miR-21 OE mice were obtained from Dr Mark Hatley, St Jude's Research Hospital, US (Hatley et al. 2010) and bred in Bristol. Transgenic *CAG-LacZ-miR21-EGFP* mice were generated by pronuclear injection according to standard protocols. A genomic sequence of the pre-miR-21 sequence was inserted into CAG-Z-EGFP vector. To over express *miR-21* expression, the *CAG-Z-miR-21-EGFP* mice were bred to *CAG-Cre* mice to yield global over-expression of miR-21 (*CAG-miR-21)* and mice carrying the transgene were identified by PCR analysis. To generate miR-21 KO mice CAG-Z-EGFP vector was inserted to replace the precursor miR-21 (pre-miR-21) sequence using homologous recombination using standard loxP and *CAG-Cre* methods as described previously (Hatley et al. 2010). Deletion of pre-miR-21was not associated with disruption of TMEM49 gene expression as evidenced by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) (Lakhia et al. 2016). Strain matched WT mice were purchased from Charles River and both the mutation and WT mice have remained inbred on the C57BL/6 strain. Both sexes male and female mice were used for different experiments.

2.2 Bromodeoxyuridine injection and detection

To determine cell proliferation and neurogenesis in the hippocampus of mice, bromodeoxyuridine (BrdU) solution (Roche) (10 mg/ml in PBS) was injected into

the peritoneal cavity of mice at a dose of 100 mg/kg. For examination of progenitor proliferation in the dentate gyrus (DG) and the subventricular zone (SVZ), mice were killed 2 hours after the injection. For migration and differentiation analysis, BrdU solution (10 mg/ml in PBS) was injected into the peritoneal cavity of mice at a dose of 100 mg/kg every two hours for three times, and mice were sacrificed three days later. For cell survival analysis, BrdU solution (10 mg/ml in PBS) was injected into mice at a dose of 100 mg/kg every day for 5 consecutive days, and the mice were killed 3 weeks later.

2.3 Histological Processing

Animals were terminally anaesthetized with sodium pentobarbital (200mg/kg intraperitoneal) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Following perfusion, the brains were harvested and post fixed in 4% paraformaldehyde in PBS at 4ºC overnight. For cryo-protection brains were then incubated in 30% sucrose in PBS at 4ºC for 4 days or until sectioning.

Brains were embedded in optimum cutting temperature (OCT) medium (Cellpath, UK) on dry ice for sectioning. Frozen brain tissues (-20°C) were sectioned either sagittally or coronally. Depending on the area of interest and applications, 40µm sections were obtained for free floating sections used for immunohistochemistry and 12.5µm sections were obtained for *in situ* hybridization (*ISH).* For tissue for quantitative real time polymerase chain reaction (qRT-PCR) and Western Blotting experiments, animals were terminated by Schedule 1 and the fresh brains were

harvested. The hippocampus was dissected on ice and stored at -80°C for RNA and protein extraction immediately until use.

2.4 Immunohistochemistry

2.4.1 Immunocytochemistry

The primary and secondary antibodies used for all experiments are summarized in Table 2-1. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then washed 3 times for 5 minutes with PBS. Cells were blocked for 1 hour at room temperature in 10% normal goat serum (NGS, Sigma) made up with 0.3% Triton X-100 (Sigma) PBS (PBS-T). Immunolabelling was performed by applying the required primary antibody, diluted in 10% NGS in PBS-T, overnight at 4°C. The following day the primary antibody was removed, and cells were washed 3 times for 5 minutes in PBS. The required secondary antibody was diluted in PBS-T, then applied for 2 hours at room temperature in the dark. Secondary antibody was then removed, and cells were washed 3 times for 5 minutes in PBS. The immunolabelled coverslips were mounted using either soft-set Vectashield mounting medium with DAPI (VectorLabs, CA, USA) to label nuclei.

2.4.2 Immunohistochemistry using DAB reagent

Free floating sections were immunolabelled in 24 well plates using the required primary and secondary antibodies listed in Table 2-1. All washes were carried out with PBS-T, three times for 5 min each time. Sections were treated with 0.3% H_2O_2 in 0.3% triton/PBS for 30 min (50 µl 30 % H_2O_2 in 5 ml 0.3% Triton/PBS), then washed again in PBS-T for three times 5 min each time. Sections were then

incubated in blocking solution (10% normal goat serum in 0.3% Triton/PBS) at room temperature for 1 hour, followed by incubation with the relevant primary antibody at 4ºC overnight (Table 2-1). The following day, sections were rinsed with PBS-T three times, followed by the incubation with appropriate secondary antibodies (Table 2-1) at room temperature for 1-2 hour. Sections were then incubated in ABC solution (Vectastain Elite kit) according to manufacturer's instructions. A final wash was performed before the incubation in 3,3′-Diaminobenzidine (DAB) and Nickel II chloride (NiCl2) solution for 1 min (Vector labs). Sections were washed in PBS for three times. Sections were mounted on Superfrost+ (ThermoFisher Scientific) slides, dried and dehydrated by serial washes in alcohol (70%, 95% and 100%) for 1 min in each, then dilipidated by two washes in xylene 2 min in each and mounted with DPX-medium (Sigma).

2.4.3 Fluorescence staining

Free-floating sections were washed 3x with PBS, for 5 min each time. For permeabilization, sections were incubated with 20% HCl for 30 min at room temperature in order to retrieve the BrdU antigen. Sections were then washed with 0.3% Triton/PBS (PBS-T) three times each time for 10 min to allow the neutralisation of the pH. Sections were then blocked in 10% NGS in PBS-T for 1 hour at room temperature. After blocking, sections were then incubated with the relevant primary antibodies (Table 2-1) diluted in PBS-Triton, containing 10% NGS and incubate overnight at 4°C. Sections were then washed with PBS-Triton three times for 5 min. Following the washes, they were incubated with the secondary antibodies (Alexa Fluor dye series) (Table 2-1) at room temperature for two hours in the dark. Free floating sections were washed 3 times with PBS and

mounted onto Superfrost+ slides (ThermoFisher Scientific), briefly allowed to dry, then mounted in Vectashield with DAPI (Vectorlab). Stained slides were kept at 4 °C in the dark.

Table 2-1: List of antibodies used, species raised in, dilutions used and suppliers.

Antibodies	**Species raised in**	**Working dilution**	**Company**
IgG anti BrdU	Rat	1:500	Abcam
IgG anti Ki67	Rabbit	1:500	Abcam
IgG anti Nestin	Mouse	1:200	Abcam
IgG anti GFAP	Rabbit	1:1000	DAKO
IgG anti Tuj1	Rabbit	1:500	Covance
IgG anti DCX	Rabbit	1:500	Abcam
IgG anti Caspase-3	Rabbit	1:200	Cell Signalling
IgG anti NeuN	Mouse	1:500	Chemicon
IgG anti Calretinin	Rabbit	1:2000	Abcam
IgG anti NG2	Rabbit	1:500	Cell signalling
IgG anti F4/80	Rat	1:200	BIO-RAD
Alexa fluor 568 anti-rat IgG	Goat	1:500	Life Tech.
Alexa fluor 488 anti-mouse IgG	Goat	1:500	Life Tech

Alexa fluor 488 anti-rabbit IgG	Goat	1:500	Life Tech
Alexa fluor 568 anti-rabbit IgG	Goat	1:500	Life Tech
Alexa fluor 568 anti-mouse IgG	Goat	1:500	Life Tech
biotinylated anti-mouse	Mouse	1:200	Vectashield
biotinylated anti-rabbit	Rabbit	1:200	Vectashield

2.4.4 Nissl Staining

Twelve µm sections of the olfactory bulb (OB) were obtained directly onto Superfrost+ slides (ThermoFisher Scientific). The sections were dried for 30 min at room temperature, washed in distilled H_2O for 1 min. They were then stained in Crestal Violet Acetate (0.2%) for 1 min and washed again in distilled water for 1 min. Following sequential dehydration steps for 1 min each in 70%, 95% and 100%, followed by two consecutive incubations in xylene for 2 min. Slides were mounted with DPX-medium (Sigma) and left to dry for 2 hours.

2.5 Microscopy

Fluorescence staining was viewed under a Leitz DMRD fluorescent microscope attached to a Leica DFC340FX digital high-sensitivity monochrome camera (1392x1040, 6.45µm pixels, 8 or 12-bit, 21 fps full frame) and visualized using Leica application suite 3.3.1. DAB stained sections were viewed under a Leitz DMRD microscope attached to a Leica DC500 42 bit-colour digital camera and visualized using the Leica IM50 4.0 software. The following Leica lenses were

used: 5x dry with a numerical aperture (NA) of 0.15, 10x dry with a NA of 0.3, 20x dry with a NA of 0.4 and a 40x oil with a NA of 1.25.

2.6 Quantification of cultured cells and sections

Cell count was performed either manually or using ImageJ (http://rsbweb.nih.gov/ij/). To quantify the number of proliferating cells in the DG, SVZ and OB, the number of Ki67+ and BrdU+ cells associated with a DAPI positive nucleus within the area of DG and SVZ respectively, was quantified in 4-6 representative sections per replicate. This was applied to the quantification of DCX+ and Calretinin+ cells, immature neurons in the DG region. To quantify the areaof DCX+ cells in the SVZ and RMS, images from equivalent SVZ regions from different treatments were analyzed with ImageJ (NIH, USA) to quantify the volume of DCX+ staining per relevant area within each image for each replicate. To quantify mature neurons (NeuN+ cell) in the DG and Cornu Ammonis (CA1 and CA3), images from equivalent hippocampal regions from different treatments were analyzed with ImageJ software (NIH, USA) to quantify integrated density of NeuN staining per relevant area within each image for each replicate. The threshold adjustments were sit to quantify only positive immunostaining and the adjustments were fixed in all measured sections.

For OB size measurement, the total surface area measurements were expressed as percentage volume of the entire OB from different treatment. For analysis of each cell layer measurements of the cell layer were normalised to the whole surface area. Specific labelling of different layers within the OB was used to measure the thickness of each cell layer. The thickness was normalized compared to the radius

of each OB. In each experiment, 3 or more mice per group were used for quantification. The acquired images were viewed in ImageJ (NIH, USA).

For differentiated NPCs quantification, cells positive for BrdU, Tuj1, NG2, GFAP and nestin (Table 2-1) were quantified with ImageJ (NIH, USA) and the average of number of positive cells in five different fields was measured in each coverslip for each treatment. At least 3 coverslips were used for each treatment. For axonal growth measurement, the average axonal length was calculated in five different fields in three coverslips for each treatment.

2.7 SDS-PAGE and Western Blotting

2.7.1 Protein extraction

The frozen tissue was homogenized in Radioimmunoprecipitation assay (RIPA) buffer containing 50mM Tris-HCl pH 7.6 (Sigma-Aldrich, U.S.A), 150mM NaCl, 0.1% Triton-X100, 0.1% Sodium deoxycholate, 0.1% Sodium dodecyl sulphate (SDS) and 1X complete EDTA-free protease inhibitor (Roche, Germany). The tissue in RIPA buffer was homogenized by passing the lysate through a 1ml Terumo 21g needle for 10 times. The homogenised samples were then centrifuged and the supernatant was stored in a RNase-free tube and kept on ice. The concentration of the extracted protein sample was measured using colorimetric protein assay, BCA assay kit protein (Pierce Technology, UK) according to manufacturer's instructions. Aliquots of 25µg of protein were stored at -80 °C until use.

Protein samples of 25µg from each group were thawed on ice and loading buffer (2X) was added to each sample. Samples were then denatured at 95°C for 5

minutes, then centrifuged briefly. The SDS-polypeptide complexes were separated on 10% polyacrylamide gels containing SDS. Each gel consists of a stacking gel layer at the top and a resolving gel layer below the stacking gel. Samples were then loaded into each well of the stacking gel, with 10µl of the Trident Prestained Protein Ladder (GeneTex). Gel electrophoresis in Tris-Glycine running buffer was performed using electrophoresis system (Bio-RAD. U.S.A) at 100 Volts for 20-30 minutes, then at 150 Volts for a further 20-30 min. Separated proteins were transferred onto a PVDF membrane (Amersham, GE Healthcare, U.K), as the gel and PVDF membrane were sandwiched between thick blot papers (BIO-RAD. U.S.A) and sponge sheets, and placed on transfer machine (Trans-Blot®, BIO-RAD, U.S.A). Four hundred mA was applied on the machine for 90 minutes to complete protein transfer to the PVDF membrane. The membrane was then blocked in 5% non-fat milk (Marvel) and incubated overnight at 4°C with the primary antibody (Table 2-2). The membrane was then washed in TBS-T before being incubated in the corresponding (HRP)-conjugated secondary antibody. All solutions used in WB are illustrated in Section (2.7.2).

Following incubation in secondary antibodies, the blots were washed in TBS-T, and incubating with the ECL reagents (Amersham, GE Healthcare). The membrane was transferred to Hyper-cassette™ Autography Cassette (Amersham) and exposed to medical Super X-Ray films (Fuji, Japan) in the dark room to visualise the bands on the film. The film was then processed using KODAK M35 X-OMAT Processor and scanned. The optic densitometry measurements were

performed using ImageJ software (NIH, Bethesda), using the mean of all three samples per mouse group. Values were expressed relative to the wildtype.

Table 2-2: List of antibodies used, species raised in, dilutions used and suppliers.

Antibody	Dilution	Species	Company
PTEN	1:500	Mouse	Cell Signalling Technologies
p-PTEN	1:500	Rabbit	Cell Signalling Technologies
AKT	1:500	Rabbit	Cell Signalling Technologies
STAT3	1:500	Rabbit	Cell Signalling Technologies
β-catenin	1:5000	Mouse	Cell Signalling Technologies
ERK	1:500	Rabbit	Cell Signalling Technologies
BCL2	1:500	Mouse	Cell Signalling Technologies
Caspase-3	1:1000	Rabbit	Cell Signalling Technologies
Spry2	1:400	Rabbit	Santa Cruz
α-tubµlin	1:2000	Mouse	Sigma-Aldrich
Horseradish peroxidase	1:10000	Mouse/Rabbit	Amersham, GE Healthcare

2.7.2 Western blot solutions

• 10 % SDS-PAGE gel (25 mL total)

- 9.9 mL Water
- 8.3 mL 30% acrylamide (Sigma)
- 6.3 mL 1.5M Tris (pH 8.8) (Sigma)
- 250 µL 10% Sodium Dodecyl Sulphate (Sigma)
- 250 µL 10% Ammonium persulfate (APS)
- 10 µL Tetramethylethylenediamine (TEMED)

• Stacking gel (8 mL total)

- 5.5 mL Water
- 1.3 mL 30% acrylamide
- 1.0 mL 1.0M Tris (pH 6.8)
- 80 µL 10% Sodium Dodecyl Sulphate (Sigma)
- 80 µL 10% Ammonium persulfate (APS, Sigma)
- 8 µL N, N, N', N'-Tetramethylethylenediamine (TEMED, Sigma)

- **Loading buffer**

  - 1 M Tris-HCl pH 6.8 (Sigma)
  - 10% SDS (Sigma-Aldrich)
  - Glycerol (Sigma)
  - β-mercaptoethanol
  - 1% Bromophenol blue (Sigma-Aldrich, U.S.A)
  - dH2O up to 10 ml

• Transfer buffer

- 4.8 mM Tris Base (Melford)
- 3.9 mM Glycine (Sigma)
- 0.0037 % Sodium Dodecyl Sulphate (Sigma)
- Water (up to 1L)

• Running buffer

- 25 mM Tris Base (Melford)
- 0.19 M Glycine (Sigma)
- 0.05 % Sodium Dodecyl Sulphate (Sigma)
- Water (up to 1L)

• Tris-Buffered Saline plus tween20 (TBS-T)

- 20 mM Tris Base (Melford)
- 20 mM Tris HCl (Melford)
- 0.15 M NaCl (Sigma)
- 1 mL Tween20 (Sigma)
- Water (up to 1L)

2.8 RNA extraction from hippocampal tissue

Hippocampal-dissected tissue was frozen immediately using dry ice to preserve RNA and stored at -80°C until use. Total RNA was extracted from hippocampal tissues using the mirVana™ Kit (Ambion®, Life Technologies) according to the manufacturer's instructions. At the end of the RNA extraction process, RNA was eluted with pre-heated 50 µl of nuclease free water to recover the RNA and used immediately otherwise kept in -80°C until use.

The concentration of total RNA in 1 µl of each sample was measured on the ND-1000 spectrophotometer (NanoDrop Technologies, UK) against water as blank and using the 50 lid for higher RNA concentrations and 10 lid for lower concentrations. The quality and integrity of the RNA were checked by the ratios of A_{260}/A_{280} measurements to measure the ratio of RNA to protein (ratios in the range of 1.8-2.00 indicate no contamination in the samples, while lower values may indicate suggesting high levels of protein within a sample).

2.9 Analysis of miRNA expression

To obtain equal amounts of RNA across samples for the reverse transcription (RT) step, a calculated amount of RNA from each sample was diluted with RNase free water to get 200 ng needed for the RT. RNA were reverse-transcribed using (microRNA reverse transcription kit, Applied Biosystem). Briefly, the RT mix 2µl of 5X RT microRNA primers (Table 2-3), 3.33µl of total RNA sample (2ng) and 6.67µl of master mix (TaqMan®) were prepared for each PCR reaction in 0.2 ml polypropylene reaction tubes. The reactions were incubated in an Applied Biosystems Thermocycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and

then at 4°C until collection. All Reverse transcriptase reactions were run in duplicate.

Table 2-3: 5X RT microRNA primers.

All primers were obtained from (TaqMan® Small microRNA Assay. ThemoFisher Scientific).

miRNA	Mature sequence (3'…->5')
miR-132	UAACAGUCUACAGCCAUGGUCG
miR-134	UGUGACUGGUUGACCAGAGGGG
miR-138	AGCUGGUGUUGUGAAUCAGGCCG
miR-128	CGGGGCCGUAGCACUGUCUGAGA
miR-212	UAACAGUCUCCAGUCACGGCCA
miR-21	UACUUAUCAGACUGAUGUUGA
RNU6B	GUGCUCGCUUCGGCAGCACAUAUACUAAAAUUGGAACGAU ACAGAGAAGAUUAGCAUGGCCCCUGCGCAAGGAUGACACG CAAAUUCGUGAAGCGUUCCAUAUUUU

For the PCR amplification step, a 1.33 µl of RT product and 18.67 µl of the qPCR reaction mix comprising 1µl of (TaqMan® Small RNA Assay 20x), 10 µl of TaqMan® Universal PCR Master Mix II 2x and 7.67 µl of water were transferred on a 96-well plate. All PCR reactions were performed using standard PCR conditions (50°C for 2 min, 95°C for 10 min, 95°C for 15 min and 60°C for 1 min for 40 cycles on the StepOne thermocycler (Applied Biosystems). The cycle

threshold (Ct value) was acquired using StepOne Software and the relative expression of the genes was determined using the 2-ΔΔCt method. RNU6 was used as the normalization gene for all the microRNAs examined.

2.10 Production of cDNA for gene expression

The SuperScript® II RT (Life technologies) was used to produce cDNA, following manufacturer's instructions. Briefly 2 µg total RNA was added to 1 µl 50–250 ng random primers, 1 µl dNTP Mix (10 mM each) 1 µl distilled water up to 12 µl 2. The mixture was heated to 65°C for 5 min, followed by a quick chill on ice. Then, 4 µl of 5X First-Strand Buffer and 0.1 M DTT were added to the mixture and it was incubated at 42°C for 50 min. Upon completion, cDNA was diluted 10-fold (1µl cDNA with 9µl dH_2O) and then stored at -20 °C until further processing.

2.11 q-PCR

Quantitative PCR (qPCR) was performed using the synthesized cDNA, using specific primers (Table 2-4), and SYBR Green PCR master mix (Applied Biosciences, Warrington, UK) in a 96-well plate. For reaction, 1µl of cDNA was incubated with 3.6 ul forward primer (100pM), 3.6 µl reverse primer, 30 µl SYBR and 20.4 µl water. 4 samples of cDNA from different individual mice per mouse group were used per plate. For the negative controls, 1µl of distilled water (dH2O) was used. The primers used per gene of interest are shown in Table 4. After loading all samples, the 96-well plate was then centrifuged briefly before being loaded into the StepOne Plus Thermocycler (Applied Biosystems). Samples underwent 40 rounds of thermocycling using standard conditions (50°C for 2 min, 95°C for 10 min, 95°C for 15 min and 60°C for 1 min). The cycle threshold (Ct value) was

obtained using StepOne Software and the relative expression of the genes was determined using the 2-ΔΔCt method.

Table 2-4: Genes investigated by q-PCR and the forward and reverse primers used.

All primers were obtained from SIGMA-Aldrich.

Gene	**Forward primer sequence (5' …-> 3')**	**Reverse primer sequence (3'…->5')**
Spry1	GAGGATTTCAGATGCATGG	TTTGATCTGGTCTAGGGAC
Spry2	CCTCTGTCCAGGTCCATCAG CACTGTCAGC	GCAGCAGCAGGCCCGTGG GAGAAG
PTEN	TTGGCGGTGTCATAATGTCT	GCAGAAAGACTTGAAGGCGTA
BCL-2	ATGACTGAGTACCTGAACC	ATATAGTTCCACAAAGGCATC
TGFR	CCTGCAAGACCATCGACAT G	TGTTGTACAAAGCGAGCACC
PDCD4	GAAATTGGATTTCCGCATCT	TAACCGCTTCACTTCCATTG
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

2.12 Fluorescent in Situ Hybridization (FISH) for microRNA and immunofluorescent staining

Fresh frozen mouse brains were sectioned at 12.5 µm. Cryosections were fixed in 4% paraformaldehyde and treated with proteinase K 5µg/ml (Sigma, UK) at RT for 10 min. The sections were acetylated in 1.356% acetic anhydride/ 0.25% triethanolamine, washed in PBS+ 0.1% Tween-20. Sections were pre-hybridised in hybridisation solution (50% formamide, 5X saline sodium citrate (SSC), 0.5

mg/ml yeast tRNA, 1X Denhardt's solution) for 2.5 h at 50°C, following which they were hybridised with a DIG-labelled probe complementary to mouse miR-21 (0.5 pmol, LNA miRCURY probe; Exiqon UK) overnight at 50°C. Scrambled probes were used as a control. Probe sequences are as follows: miR-21 TCAACATCAGTCTGATAAGCTA;
scrambled GTGTAACACGTCTATACGCCCA. Following hybridisation, sections were washed twice with 60°C SSC for 10 min, then washed twice with 0.2 X SSC at 60°C for 30 min each with agitation. Sections were then washed twice in (PBS+ 0.1% Tween-20, 60°C) for 10 min each and incubated at room temperature with Western blocking solution for 1 hour (Roche, UK). Sections were incubated with a mouse anti-DIG horseradish peroxidase antibody (1:500, Abcam, UK) in a humidified chamber at 4°C overnight. Following three washes in PBS + 0.1% Tween-20 for 10 min each at RT, sections were incubated in 10% normal goat serum (Sigma, UK) at room temperature for 1 hour, followed by antibody incubation at room temperature for 4 hours (Table 2-1), washed three times with PBS + 0.1% Tween-20, and incubation with the secondary antibody Alexa-568 goat anti-rat at room temperature for 1 hour (1:500, Life Tech, UK). Slides were then washed four times in PBS + 0.1% Tween-20 for 10 min each at RT. The *in-situ* hybridisation signals were enhanced using a tyramide amplification system labelled with Alexa-488 according to the manufacturer's instructions (Invitrogen, UK). Slides were washed 3 times with PBS + 0.1% Tween-20 and mounted in Vectashield mounting medium with DAPI (Vector Labs, UK) before being analysed with a fluorescent microscope (Leica DMRB, UK).

2.13 Culture of neural stem cells from the subventricular zone (SVZ)

Adult SVZ-NPCs were harvested from 8–10 week-old mice as described previously (O'Keeffe et al. 2009). Tissue from 4–5 mice was pooled for each culture. Adult (8-10 weeks old) male mice were killed by cervical dislocation, and their brains were immediately removed. The brains were placed in ice cold serum-free DMEM/F-12 medium under a dissection MZ6 light microscope. Under the microscope the cerebellum and the olfactory bulbs were removed, and the meninges were discarded. The remaining part of the brain was cut in three blocks by two coronal sections, one caudal to the hypothalamus and one rostral to the optic chiasm. The SVZ of the lateral ventricles (≈ 1 mm from the lumen) was carefully collected and treated with 0.1% [v/v] trypsin (Worthington Biochemical) for 7 min at 37 °C. Digestion was stopped with a mixture of 0.1% [v/v] trypsin inhibitor and 0.008% [v/v] DNase (Worthington Biochemical) at 37 °C for 5 min. The cells were gently triturated and sieved through a 40 μm-cell nylon cell strainer (BD Biosciences). 2 x 10^5 cells/ml were grown in DMEM: F12 (3:1), B27 (2%), EGF plus FGF-2 (20 ng/ml of each), 5 μg/ml Hep, 100 U/ml penicillin, and 0.1 g/l streptomycin (Sigma). Primary SVZ-NPCs were expanded for 10 days at 37°C in humidified 5% CO_2 to permit neurosphere formation. A minimum cut off of 200μm diameter was used to define a neurosphere.

To label dividing cells, 0.2 μM BrdU (Sigma) was added to NPC-cultures, 72 h before plate-downs. Before plating, neurospheres were collected, dissociated and resuspended in DMEM: F12 (3:1) and 2% B27 supplemented with 1% fetal calf serum (FCS). 5 × 10^4 cells were seeded on the middle of each coverslip and left in

the incubator for further 30 min to allow cells to attach. 500 µl of DMEM: F12 (3:1), 2% B27, and 1% FCS were added to each well. Half of the medium was replaced with fresh media after 3–4 days, if needed. Cells were allowed to differentiate for seven days before fixation with 4% PFA. The workflow of adult SVZ culture experiment is illustrated in Figure 2-1.

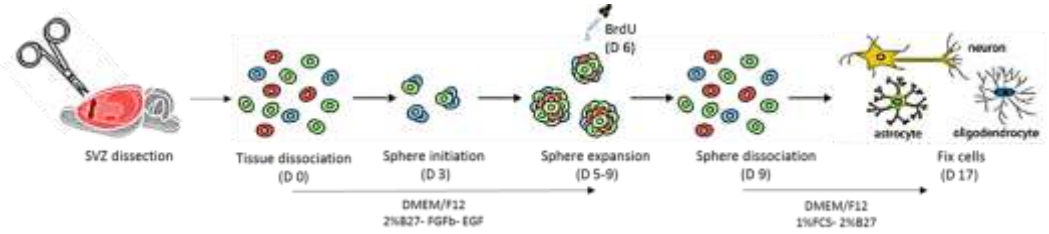


Figure 2-1: The workflow SVZ culture experiment.

Adult SVZ was dissected, dissociated and culture with growth factors to stimulate cell proliferation. BrdU was added on day 6 of culture to label proliferating cells, and spheres were dissociated and allowed to differentiate for another 7 days. The percentage of different cell lineages was measured compared to proliferating cells.

2.14 Behavioural Experiments

2.14.1 Water maze

The apparatus and behavioural procedures have been previously described (Vorhees & Williams 2006). Water maze test was conducted in a 1.5m-diameter circular tank (60 cm in height and 180 cm in diameter) filled to a depth of 40 cm with water made opaque by adding white, non-toxic paint. Water temperature was maintained at 19-20°C. The pool was divided into 4 quadrants and a movable escape platform (12cm diameter) was submerged 0.5 cm below the water surface, in a fixed position for the training period. The pool was placed in a dimly lit room

with fixed distal visible cues that served as navigational reference key for locating the ending target. Before the start of the task, a visible platform test for male mice participants was conducted to test for their vision and motor skills. Mice were trained over 5 days. On each training day, mice received 4 training trials with a 30 s interval between each session. On each trial they were placed into the pool, facing the wall, in 1 of 4 start locations. The order of these start locations was randomly varied throughout training. The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the mouse was guided onto the platform. The escape latency was calculated as the average of total time taken in all trials for each animal on each day of the acquisition and reverse task to locate the platform and was used as a measure for spatial learning. Following the completion of training, spatial memory was assessed by a probe test (retrieval test) to test for memory consolidation. In this test the platform was removed from the pool, and the mouse was allowed 60 s to search for it. The frequency of passing through the target quadrant and the time spent swimming in the target quadrant searching for the removed platform was recorded. Following the training test, a reversal training test was performed, using the same method, but the platform was located in the opposite position to the training test. The procedure was recorded using a digital video tracking system (View point behaviour Technology).

All data analysed by ANOVA were normally distributed. Tukey's ***post hoc*** test was used to identify differences between groups. Behavioural data from the training and reversal period were analysed using repeated measures ANOVA. Data from the probe test, speed and visible platform trials were analysed using one-way

ANOVA. Data from the visible platform test were analysed using one-way ANOVA. All behavioural data were analysed in GraphPad prism® (version 5.03).

2.15 Bioinformatics analysis

To identify predicted targets and the signalling pathways for miR-21 in adult neurogenesis, three microRNA bioinformatics programmes were used. Each of these programs use different algorithms to generate potential miR-21-5p targets. The bioinformatics programs used were TargetScan (release 7.1) (Agarwal et al. 2015), DIANA TarBase (version 7.0) (Vlachos et al. 2015) and miRDB (Wong & Wang 2015a). In all cases, the predicted genes were sorted based on their cumulative score. Only predicted gene targets for mmu-miR-21a-5p with conserved binding sites were used for further analysis. DIANA TarBase and TargetScan generated genes which were predicted using the algorithm and experimentally validated. TargetScan, DIANA and miRDB generated a list of genes. That included many transcript variants of genes. The results were ranked by the highest scoring and the highest scoring transcript variant was kept while the rest were removed. More details are provided in Chapter 4.

The generated targets were then input into STRING, a protein-protein interaction database, to associate the generated targets with Kyoto Encyclopaedia of Genes and Genomes (KEGG) biological pathways, measured using the False Discovery Rate.

2.16 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6. For immunohistochemistry, immunofluorescence, Nissl-staining, Western blot and q-

RT – PCR results, one-way ANOVA was used followed by Tukey's multiple comparison test as a *post hoc* analysis. Statistical significance was determined at $p<0.05$. Values given in the text and figures represent the mean and standard errors. Appropriate sample sizes (n) were used, depending on the experiment and are described in the relevant figures.

Chapter 3 Role of miR-21 in neurogenesis within the adult dentate gyrus

3.1 Introduction

Neurogenesis in the adult brain is a continuous process throughout life. This process in the adult brain is restricted to two regions: the sub-ventricular zone (SVZ) within the lateral ventricles and the sub-granular zone (SGZ) within the hippocampus. In both areas newly generated neurons integrate into the existing local neural network, and then become indistinguishable from neurons that are generated during development (Kee et al. 2007; Trouche et al. 2009). The ability to add new neurons into the mature hippocampal structure contributes a unique form of plasticity in the memory process, as these new neurons contribute to hippocampus-related memory (Deng et al. 2010). These newly generated neurons in the dentate gyrus (DG) reside in the granule cell layer (GCL) and constitute 10-15% of the total DG population. This percentage increases with age, to reach the highest levels of newly generated neurons in 2-month-old mice (Apple et al. 2017). This increased neurogenesis is reflected in the density of the DG and Cornu Ammonis (CA3) regions that DG neurons send their projections to. However, the rate at which neurons are added to the DG reaches a plateau and then starts to decline with age, with about a 75% decrease in the numbers of proliferating progenitors at 7-9 months compared to younger animals (2 months) (Apple et al., 2017; Kuhn et al. 1996).

The process of adding new neurons and their survival is regulated by a number of influences, including extrinsic and intrinsic factors. Recently miRNAs have emerged as a new regulator for adult neurogenesis. miRNAs are a group of small noncoding RNAs (~22 nt), and they have been found to be involved in the post

transcriptional regulation of their targeted proteins, by binding to their complementary seed region in protein-encoding mRNAs (Bartel 2004; Flynt & Lai 2008). This therefore suggests their potential to manipulate the local transcriptional networks (Flynt & Lai 2008). miRNAs mostly alter their expression during developmental transition and following injury or stress (Ge et al., 2014; Krichevsky et al. 2003a; Harrison et al., 2016b; Redell et al. 2011; Sun & Shi, 2014). Additionally, a number of miRNAs have already been identified that play an important role in the regulation of proliferation and differentiation of stem cells, including neural stem cells (Han et al., 2016; Jin et al., 2016; Shenoy & Blelloch, 2014). MiR-21 is found to be involved in maintaining vascular and blood brain barrier (BBB) stabilization, with the reduction of traumatic brain injury (TBI)-induced loss of tight junction proteins (Ge et al., 2015). Additionally, miR-21 is considered to be a pro-survival miRNA and contributes to the reduction of neuron sensitivity to apoptosis (Buller et al. 2010; Buscaglia & Li 2011). Therefore, we wanted to address the function of miR-21 in relation to neuronal proliferation, differentiation and survival in the normal adult brain, and whether it has an influence on cognitive functions.

3.1.1 Hippocampal anatomy and connectivity

The hippocampus in primates is a small organ situated within the brain's medial temporal lobe; in rodents it is comprised of a larger organ under the cortex. The neuronal arrangements and pathways within the hippocampal formation are very similar in all mammals (Andersen et al. 2007). The hippocampus forms the main structure in the brain that is involved in long term memory and spatial navigation. It is comprised of two structures: the dentate gyrus (DG), and the hippocampus

proper or the ram's horns or Cornu Ammonis (CA) which is used to name the subfields of the hippocampus CA1, CA2, CA3 and sometimes CA4, and the subiculum (Figure 3-1) (Amaral et al. 2007).

DG granule cells are the principal excitatory neurons of the DG, as each DG contains about 250,000-500,000 granule cells (Kempermann et al. 1997). They receive the majority of their inputs from cortical and subcortical structures through the entorhinal cortex (EC). The EC acts as the main structure for unidirectional signal transmission between the hippocampus and neocortical areas (Wei Deng et al. 2010). Axons from the pyramidal cells in the EC mainly project to the granular layer in the DG through the perforant path, while some axons project to CA3 of the hippocampus (Deng et al. 2010). Information in the DG is projected to the dendrites of the CA3 pyramidal neurons via mossy fibres. Schaffer collaterals within the CA3 extend from the CA3 region to CA1, and from the CA1 axons, they project back to the EC and Subiculum to complete the trisynaptic circuit (EC -> DG -> CA3 -> CA1), as illustrated in Figure 3-1. Most of the hippocampal and cortical communications are through the EC, and damage to either of these regions or their associated pathways can develop into a loss of ability to create new memories, and/or recall recent past memories (Anterograde Amnesia) (Aggleton & Saunders 1997).

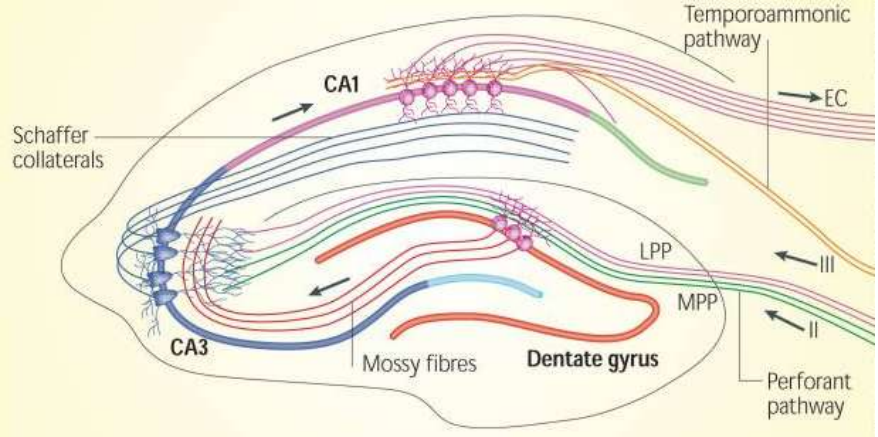


Figure 3-1: The hippocampus and network circuit.

Illustration of the hippocampus circuitry. The mature DG cells send axonal projections to the CA3 pyramidal cells through mossy fibres, and CA3 pyramidal cells send projections to CA1 pyramidal cells through Schaffer collaterals. The EC receives projections from CA1 pyramidal cells via the temporoammonic pathway (TA), and from CA3 pyramidal cells via the perfoant pathway (PP) including the lateral perforant pathway (LPP) and medial perforant pathway (MPP). Additionally, the DG send projections to the mossy fibres and receives inhibitory and excitatory projections from the mossy fibres back to the DG (Deng et al. 2010).

3.1.2 Identification and Quantification of Neurogenesis

Stem cells have the ability to proliferate and the capacity to produce different lineages. NPCs are able to increase their population by the re-entry to cell cycle. Therefore, the ability to identify proliferating cells, detect changes in neurogenesis under different conditions and the study of cell cycle activity, are important components to investigate neurogenesis. The detection of proliferating neural precursor cells has been made possible by the use of tritiated thymidine ([3H] thymidine), which was initially used to investigate cell genesis and migration in the developing brain (Altman & Das 1965). However, the complexity and limitations of using [3H] thymidine have led to the need to develop other methods of detecting neurogenesis. Bromodeoxyuridine (BrdU), is a synthetic analogue of thymidine, that can incorporate into the DNA, and substitute for the thymidine

during DNA replication. The use of BrdU with a monoclonal antibody, has enabled the labelling of the dividing cell and identification of the time and origin of neurons in all species using immunohistochemistry (Miller & Nowakowski 1988). For example, the cell cycle in mice is 24 hours and the S phase is 8 hours. Intraperitoneal injection (IP) of BrdU and sacrificing animals at different time points enables the determination of cell proliferation in the case of short term (2 hours) administration and cell survival in the case of long term (days- up to several weeks) administration to determine cell migration and survival. Co-staining tissue with BrdU and different neuronal endogenous markers enables the identification of neurons at different developmental stages, including precursor neurons and later immature and mature neurons including DCX, Calretinin, NeuN, and Calbindin. Both the migratory marker Doublecortin DCX and the calcium binding protein Calretinin are expressed by immature neurons, whereas the neuronal nuclear antigen NeuN and the calcium binding protein Calbindin CB are expressed in mature neurons.

Cell cycle re-entry can be better addressed by the use of different sequential administration of thymidine analogs. Terminal alkyne-bearing nucleotide such as 5-Ethynyl-2´-deoxyuridine (EdU), and halogenated nucleotides such as 5-iodo-20 -deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU), are examples of other thymidine analogs. These thymidine analogues function similarly to BrdU, but when they used together can be discriminated by immunohistochemistry to quantify cell cycle re-entry (Podgorny et al. 2017).

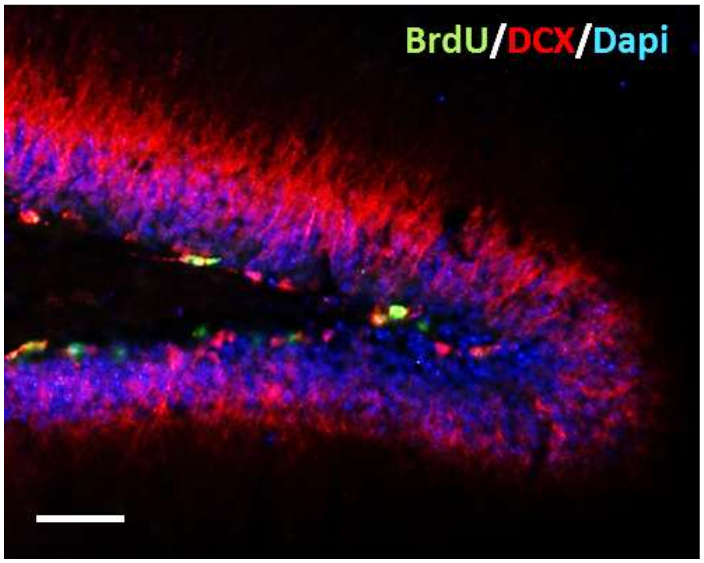


Figure 3-2: BrdU labelling of adult-generated dentate granule cells.

Adult-generated cells in the SGZ and inner GCL of the DG are visualised by immunohistochemical staining of proliferating cells BrdU (green), immature neurons DCX (red), nuclei blue (DAPI) counterstain (scale bar = 50 µm).

3.1.3 Altering neurogenesis impacts on learning and memory

The hippocampus is an important brain structure that plays a major role in the process of converting short-term memories into long-term memories, by inducing plastic changes in the strength of certain synaptic connections (Kempermann et al. 2015). In the brain, memories are not stored in one region, but they are stored based on their type in interconnected different brain regions. There are mainly two forms of memories: short-term or working memories and long-term memory; both forms can be weakened because of age or pathogenesis. The hippocampus is mainly involved in storing episodic memories, which is linked to long term memory. This function was first identified after the removal of hippocampus of an epilepsy patient named Henry Molaison (Scoville & Milner 1957). After the operation the patient lost his ability to form episodic memories and store new memories,

although his motor memories were not impaired. This indicated that although the hippocampus is crucial for storing memories, it is not associated with the storage of motor memories and permanent memory.

It has been estimated that about 1-5% new GCs are added to the mouse DG daily (Cameron & McKay 2001; Kronenberg et al. 2003). By the age of 30 days, 60% of these newly added adult-born neurons can form synapses (Toni & Sultan 2011) and contribute to global brain function based on their abilities to encode information and interact with the existing neural circuit (Ming & Song 2011). In addition, these newly added neurons have unique functions compared to the existing mature neurons, as immature neurons have a lower LTP threshold relative to mature neurons which results in the ability to encode memories from very similar stimuli (Clelland et al. 2009). The connection between DG cells and the CA3 pathway has been associated with memory acquisition and consolidation of spatial memory in the Morris Water Maze (MWM) task (Lassalle et al. 2000; Florian & Roullet 2004).

Although the link between increased adult neurogenesis and improved cognitions is well established throughout the literature, the role of newly generated neurons in the adult brain is not clear. Behavioural tasks have been developed to functionally dissect the contributions of these new neurons to learning and memory. These behavioural paradigms include the MWM, eight-arm Radial Maze (RAM), and Barnes Maze tasks, as well as the working memory test using delayed matching to sample (DMS) or delayed nonmatching to sample (DNMS) protocol to measure subsequent learning and memory performance (Creer et al. 2010;

Snyder et al. 2011; Yu et al. 2017). Accumulating evidence suggested that adult neurogenesis is required for some hippocampus-dependent tasks, and that it is not required for tasks that do not depend on hippocampus. For example, one study assessed the effects of neurogenesis ablation on spatial tasks such as water maze, contextual fear conditioning and reactivity to novel subjects in transgenic mice that over expressed the pro-apoptotic protein Bax (Dupret et al. 2008). Inhibition of neurogenesis did not affect reactivity to novel subjects as it did not result in delaying habituation. However, contextual fear conditioning task showed behavior impairment, as mice failed to show freezing fear response associated with the electric shock. Additionally, the spatial memory WM task showed navigation impairment, as mice failed to learn the hidden platform location (Dupret et al. 2008). This indicated that adult neurogenesis inhibition results in spatial memory impairment. Therefore, in summary mice impaired in their adult neurogenesis showed deficits in learning and memory tasks (Dupret et al., 2008; Jessberger et al. 2009; Yau et al. 2015).

3.1.4 miRNA in neurogenesis

Adult neurogenesis contributes to hippocampal function in the brain; these adult new-born neurons have unique abilities as they can interact with the existing neural network and respond to the local microenvironment niche. The process of hippocampal adult neurogenesis is complicated and regulated by intrinsic and extrinsic factors in the local neuronal niches, in addition to other environmental factors (for example stress, environment enrichment). miRNAs have been linked to adult neurogenesis in both the hippocampal and the SVZ (Cheng et al. 2009;

Han et al. 2016; Jin et al., 2016). For example, recently the miRNA miR-19 has been shown to regulate the migration and maturation of new-born neurons in granule cell layers of the DG by suppressing Rap guanine nucleotide exchange factor 2 (Rapgef2) (Han et al. 2016).

MiR-21, our miRNA of interest, is upregulated in many types of human cancers, and after traumatic brain injury TBI and spinal cord injury SCI. In TBI and SCI, the upregulation of miR-21 is beneficial because of it can increase cell survival which may result in alleviating brain edema and stimulating recovery in injured areas (Bhalala et al., 2012; Hu et al., 2015). No previous work has studied miR-21 functions in the normal brain, we hypothesised that loss of miR-21 will impair adult neurogenesis. This may happen through the reduction in the number of adult newborn neurons in the DG, either by reducing the proliferation and/or by impairing the differentiation fate of NPCs. Thus, to investigate this hypothesis we used transgenic mice that over-expressed miR-21 (CAG/LacZmiR-21) and mice with a knock out mutation in the miR-21 TMEM49 gene (miR-21 KO), in comparison with the normal expression of miR-21 (WT).

3.1.5 Aims

The aims of this chapter are the following:

- To investigate the effects of miR-21 overexpression or loss on the population of GCs in the hippocampus, including proliferation, differentiation and survival

- To investigate the functional consequences of miR-21 overexpression or loss in hippocampal-dependent behavioural tasks
- To investigate possible down-stream targets of miR-21 in the hippocampus

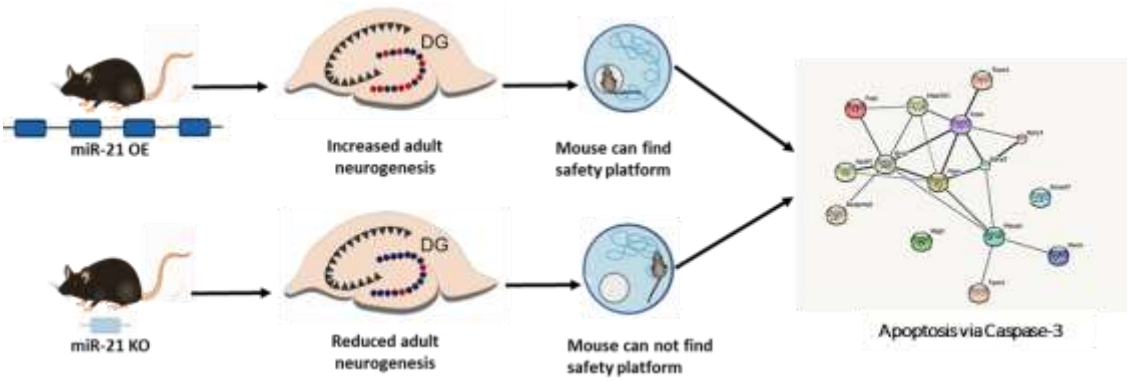


Figure 3-3: Study design and schematic of expected results.

Adult transgenic miR-21 KO mice exhibited reduced hippocampal adult neurogenesis and impaired spatial navigation skills, while miR-21 OE increased hippocampal adult neurogenesis and improved spatial navigation skills. miR-21 may act through the regulation of apoptotic activity of Caspase-3.

3.2 Results

3.2.1 miR-21 is expressed in the normal adult brain.

In order to investigate miR-21 functions in adult neurogenesis, we have used transgenic mice from Dr Mark Hatley (Hatley et al., 2010). CAG-miR-21 transgenic mice have a global knockout of the miR-21 gene (miR-21 KO) and global over-expression of the miR-21 gene by 4-6 fold (miR-21 OE). ISH showed that low expression of miR-21 in WT mouse brain, especially the neurogenic areas (Figure 3-4A-B). In addition, miR-21 was detected by qPCR in both WT and miR-21 OE mice, with an absence of miR-21 expression in miR-21 KO mice (Figure 3-3C). The miR-21 OE and miR-21 KO transgenic mice were normal and fertile and did not develop cancer even as older age mice in either the original study group or in our breeding colonies (Hatley et al., 2010).

Transgenic mice that overexpressed miR-21 or miR-21 KO mice appeared phenotypically normal, with normal fertility and no obvious gross abnormalities. To further investigate brain development, total brain weight was measured and found no significant differences between the different groups of animals (Figure 3-4D, F). The thickness of the cortical layers within the brain of miR-21 OE and miR-21 KO mice was also measured and found no significant difference between the different groups of animals (Figure 3-4E, G). Total body weight was similar across all groups (Figure 3-4H). These measurements showed no differences compared to WT animals, indicating that altered miR-21 expression in the adult brain had no effects on brain and body growth.

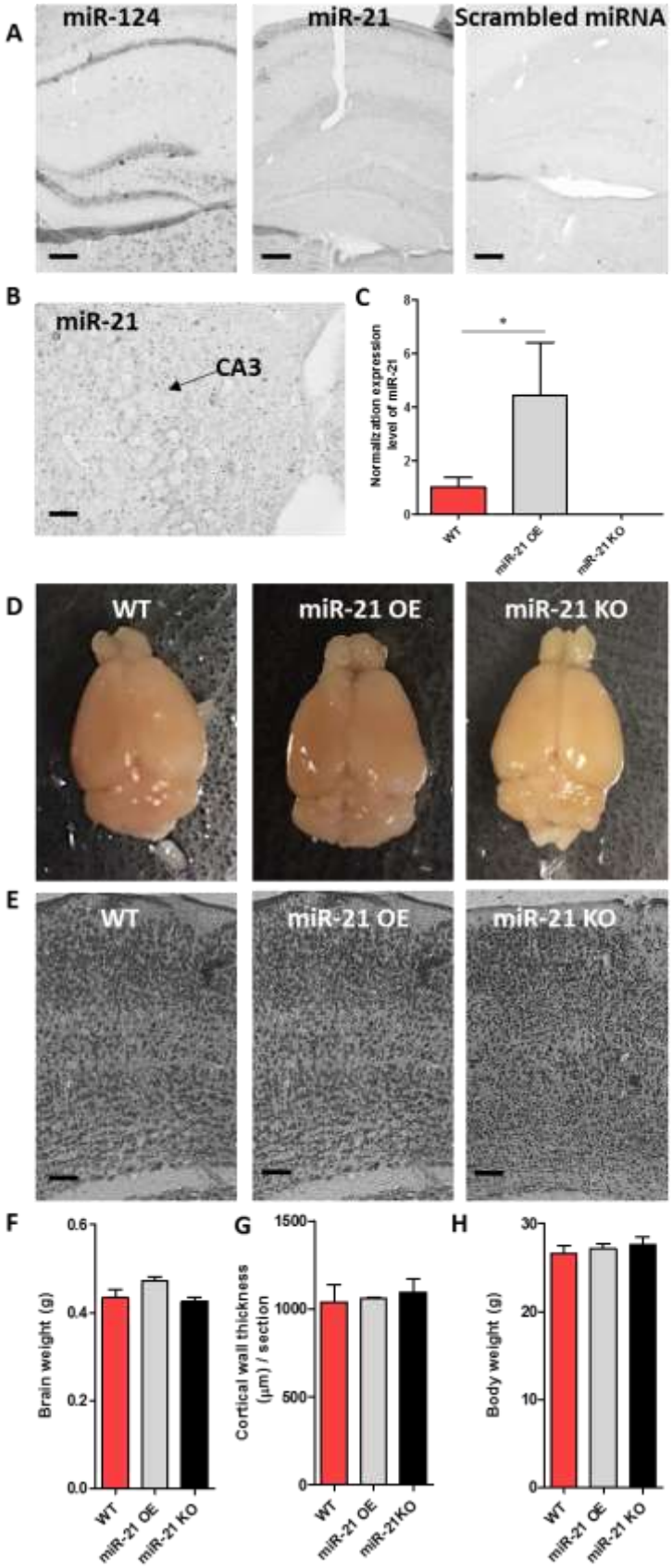

A
miR-124
miR-21
Scrambled miRNA
B
miR-21
CA3
C
Normalization expression level of miR-21
*
WT
miR-21 OE
miR-21 KO
D
WT
miR-21 OE
miR-21 KO
E
WT
miR-21 OE
miR-21 KO
F
Brain weight (g)
WT
miR-21 OE
miR-21 KO
G
Cortical wall thickness (µm) / section
WT
miR-21 OE
miR-21KO
H
Body weight (g)
WT
miR-21 OE
miR-21 KO

Figure 3-4: miR-21 is expressed in the normal adult brain.

(A) Representative images showing abundant miR-124 high expression and miR-21 expression compared to the negative control, Scrambled miRNA. Scale bar=100 µm **(B)** higher magnification showing miR-21 signal. Scale bar=50 µm. **(C)** Validation of transgenic mice, miR-21 levels in miR-21 OE and miR-21 KO compared to WT, adult hippocampus, PCR reactions were performed in triplicate and normalized to RNU6B gene. **(D)** The whole brain image of miR-21 OE and KO mice showed normal brain morphology, compared to WT brain. **(E)** Representative images showing the cortical wall for miR-21 OE and KO compared to WT. Scale bar= 100 µm **(F)** Statistical analysis for the transgenic mice body and brain weight showed no difference between groups, and measurement of the thickness of the cortical wall of miR-21 KO and OE mice did not display differences compared to the WT. (n=6, mean ± SEM, one-Way ANOVA).

3.2.2 miR-21 is localized with some of the GFAP+ cells in the hippocampus

To examine the cell type-specific localization and to further understand the regulatory function of miR-21 in the normal brain, we performed fluorescent *in situ* hybridization (FISH). Preliminary results indicated no fluorescent signal using scrambled probe, confirming our FISH specificity (Figure 3-5A). Previously it was reported that miR-21 was expressed in neurons, astrocytes, microglia in the hippocampus region (Ge et al. 2014). Additionally, miR-21 was found to be expressed in neurons and macrophage in simian immunodeficiency virus induced CNS disease, with increased expression in macrophages compared with normal brain through the secretion of miR-21 by extracellular vesicles (Yelamanchili et al. 2015). In our experiments, we detected miR-21 expression in the DG. Some of these miR-21 positive cells co-localised with GFAP positive cells, suggesting miR-21 expression in cells of the astrocytic origin (Figure 3-5B). However, we were unable detect co-localisation of miR-21 with neurons or microglia (Figure 3-5C).

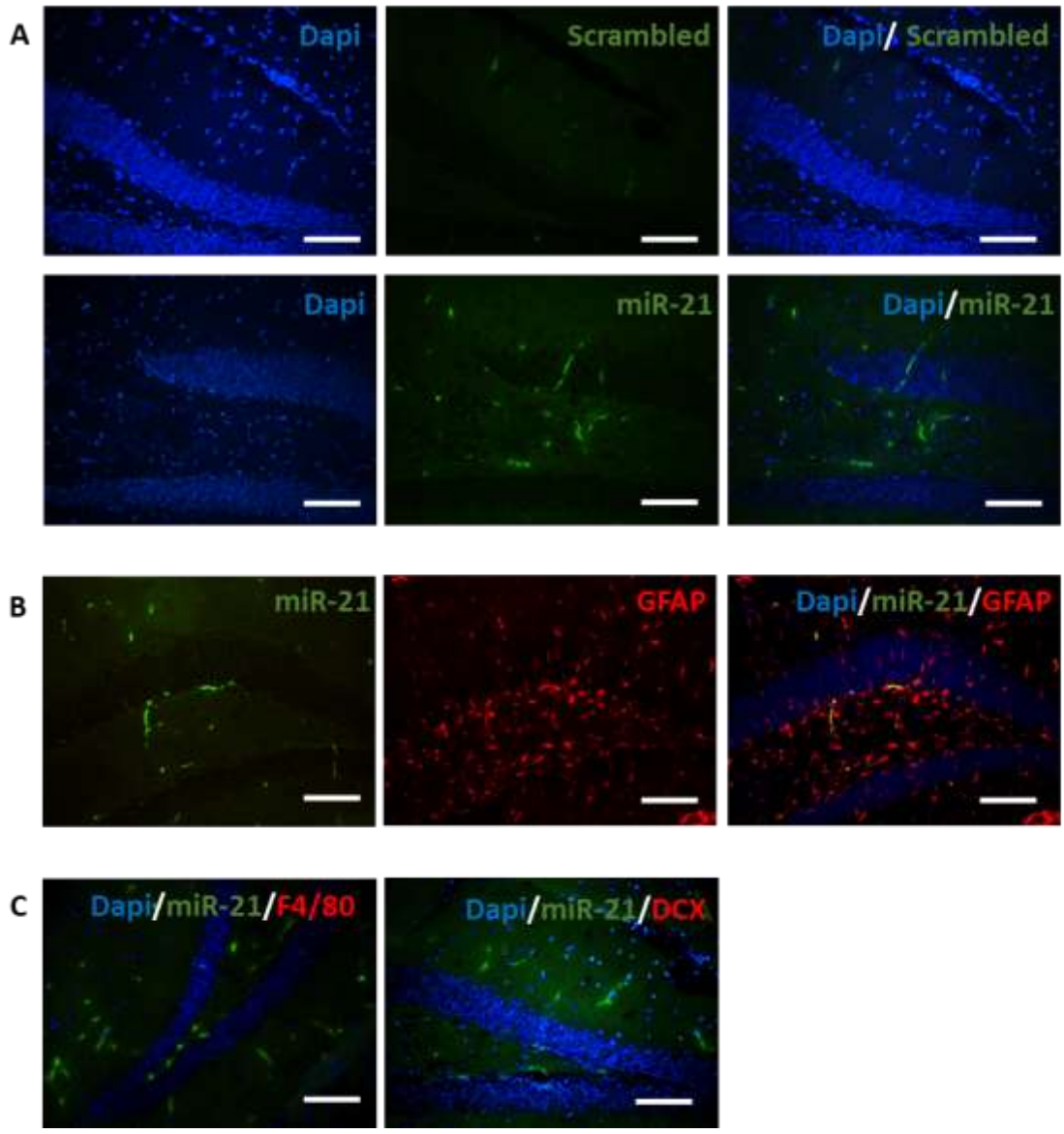


Figure 3-5: miR-21 is expressed in the normal adult brain.

(A) Representative immunofluorescent images showing miR-21 expression compared to the negative control, Scrambled miRNA. **(B)** Representative immunofluorescent images showing the localisation of miR-21 expression with some of the GFAP+ cells in the hippocampus of normal brain. **(C)** Representative immunofluorescent images showing miR-21 expression, with no immunofluorescent staining for F4/80 representing microglia or DCX representing immature neurons. Scale bar=50 µm

3.2.3 Altered miR-21 levels did not affect proliferation or astrogenesis in the hippocampus

Because miR-21 was expressed in the hippocampus, we predicted that miR-21 may involve in an important role in NPC regulation and possibly hippocampal neuroplasticity. In order to investigate the existence of adult neural progenitors in the hippocampus and to determine whether there was any change in cell proliferation, we performed short term labelling of proliferating cells using Bromodeoxyuridine (BrdU). BrdU (10mg/ml) was delivered by intraperitoneal (i.p.) injection two hours prior to harvesting of brain tissue. Cryopreserved sections were stained for the markers BrdU that labels cells at the S phase and Ki67 that labels cells at the G1, S, G2, and M phases of the cell cycle (Figure 3-6A-C). Examination of sections from transgenic mice showed no significant differences in the number of proliferating (BrdU+ and Ki67+ cells) in the dentate gyrus (DG) of the hippocampus between the different groups compared to WT mice ($p= 0.7524$, Figure 3-6D). These results indicated that altered miR-21 expression does not affect proliferation of neural progenitors.

Neural progenitor cells (NPCs) in the hippocampus can differentiate cells of the neural lineage, namely neurons or astrocytes. In order to assess the differentiation of NPCs into astroglia, we stained for Glial fibrillary acidic protein (GFAP), a marker for astrocytes, and quantified the number of GFAP+ cells in the hilus of the DG of miR-21 KO and miR-21 OE mice. Examination of brain sections from transgenic mice revealed that the number of GFAP+ cells in miR-21 OE and miR-21 KO brains were not significantly different compared to those of WT controls

(Figure 3-5E). These findings suggest that miR-21 does not have a significant role in NPC differentiation to astrocytes.

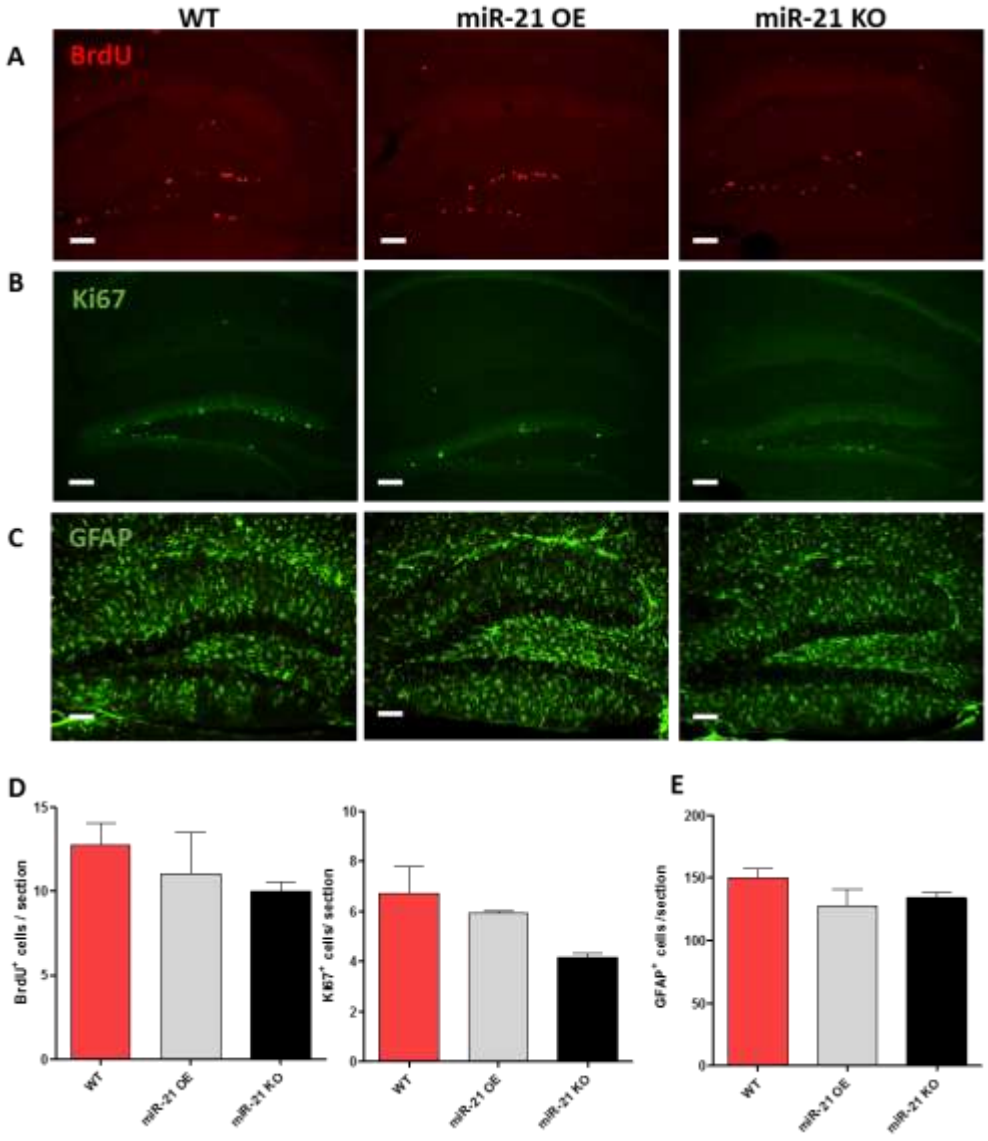


Figure 3-6: Altered levels of miR-21 does not affect neural progenitor (NPC) proliferation or astrogenesis in the adult hippocampus.

(A-B) Immunofluorescence using BrdU (red) and Ki-67 (green) markers to detect proliferation of NPCs in hippocampus of miR-21 OE and KO mice compared to WT mice. (C) Immunofluorescence using astroglial marker GFAP (green) showed no difference in miR-21 OE and KO compared to WT hippocampus. Scale bar= 100 µm. **(D-E)** Statistical analysis showed no difference in the number of Ki67+ cells and BrdU+ cells in miR-21 OE and KO compared to WT, and in the number of GFAP+ cells between the three groups of animals. (n=6, mean ± SEM, one-Way ANOVA).

3.2.4 Loss of miR-21 reduces neurogenesis in the adult hippocampus

The dentate gyrus (DG) is subdivided into the molecular layer (Mol), granule cell layer (GCL,), subgranular zone (SGZ) and hilus (H). In the DG, cells are born as type-1 radial glia-like stem cells and slowly divide to produce type-2 cells in the SGZ. Rapidly dividing type-2 cells differentiate into immature neuron type-3 cells that are Doublecortin (DCX) positive and migrate a short distance to the GCL and become Calretinin positive, where they incorporate there as mature granule cell neurons (NeuN+ cells) (Yao et al. 2012).

Despite the fact that there were no differences in proliferating cells in the DG, another mechanism by which miR-21 can contribute to neurogenesis is to promote the survival of NPCs. Thus, we assessed for immature (7-10 days old) neuronal production in miR-21OE, miR-21KO and WT mouse brains using the progenitor cell marker doublecortin DCX (Figure 3-7A). To our surprise, we observed that the number of DCX+ cells in the DG was reduced in the miR-21 KO mice compared to WT mice ($F_{(2-6)}$= 139.4, P<0.0001, Figure 3-7E). Additionally, quantification of DCX+ cells in miR-21 OE animals showed a significant increase compared to WT animals ($F_{(2-6)}$=139.4, P<0.0001, Figure 3-7E). This suggested that KO of miR-21 negatively regulated neurogenesis, while overexpression of miR-21 increased neurogenesis.

Calretinin is another neuronal marker that is expressed at later immature stages during neuron development, and we sought to validate our DCX results using Calretinin, another immature neuronal marker (Figure 3-7B). The number of Calretinin cells in the hilus of miR-21 KO mice was significantly decreased

compared to the WT ($F_{(2-8)}$=18.91, P=0.0021, Figure 3-7F), thereby supporting our DCX results and indicating that miR-21 regulates immature neuron production.

To further extend these findings, we assessed mature neuron production using the mature neuronal marker NeuN. Consistent with changes in the number of neural progenitors observed using DCX and Calretinin staining, quantification of NeuN+ cells in the DG of transgenic animals showed a reduction in the number of NeuN+ cells in miR-21 KO compared to WT ($F_{(2-6)}$=11.18, p<0.05, Figure 3-7G). However, although there was an increase in the number of NeuN+ cells in miR-21 OE compared to the WT, results failed to reach significance ($F_{(2-6)}$=11.18, p=0.1452, Figure 3-7G). We also assessed the size of the hippocampus by measuring the area of NeuN+ staining in the hippocampus. MiR-21 KO mice showed a significant reduction in the volume of DG ($F_{(2-19)}$=26.06, P<0.05, Figure 3-7H). Because increased neurogenesis may have a stimulation effects on other sub-regions, the volume of CA1/3 regions of the hippocampus was measured (Kempermann et al. 1997). There was a significant reduction in the volume of the CA1/3 region, compared to WT mice ($F_{(2-9)}$=45.76, P<0.05, Figure 3-7H). The area of NeuN+ staining in the DG and CA1/3 hippocampus was also increased in miR-21 OE mice compared to WT mice ($F_{(2-9)}$=45.76, P<0.001, Figure 3-7H), This indicated that altered miR-21expression affects adult neurogenesis by regulation the survival of immature neurons.

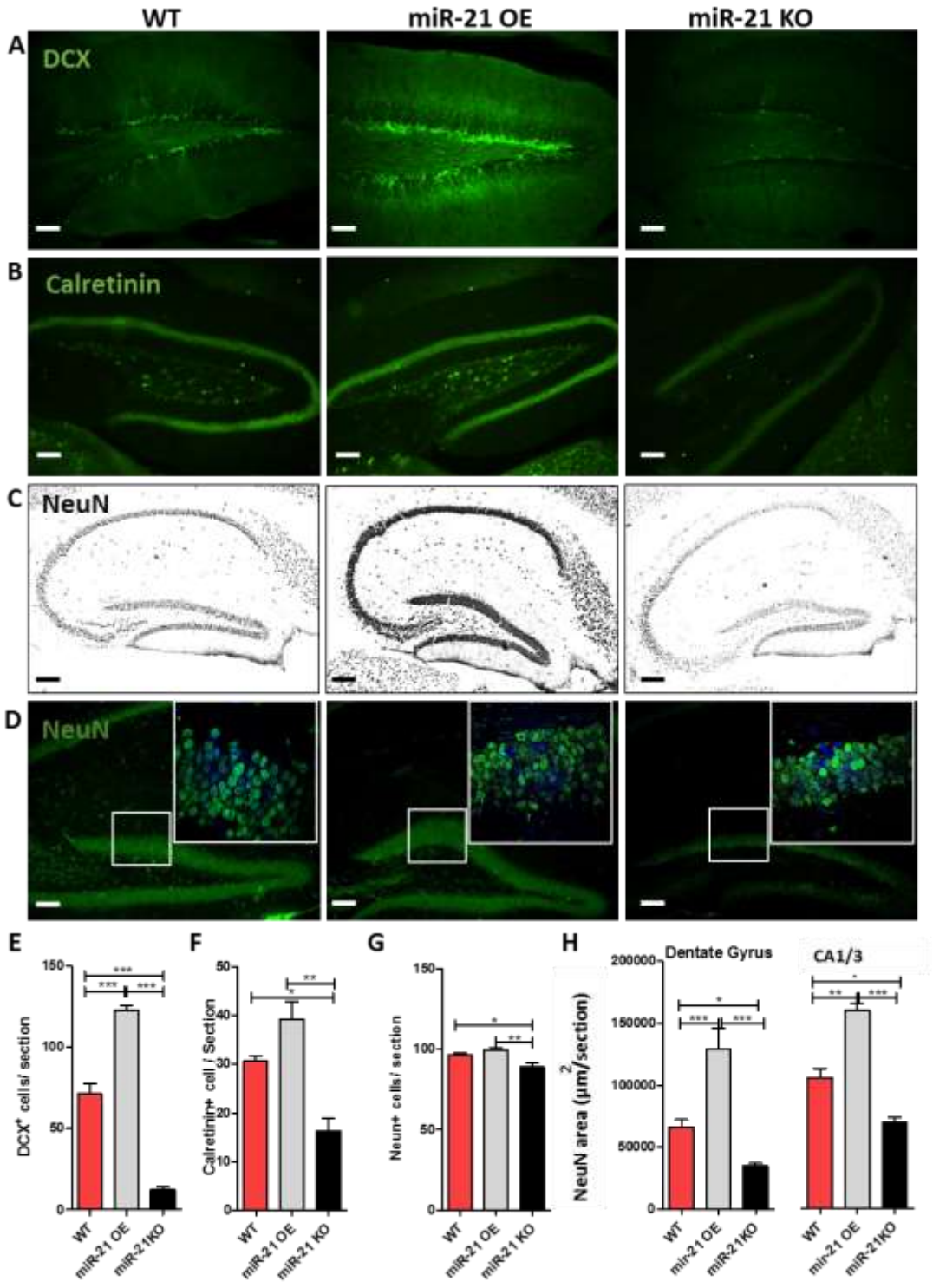


Figure 3-7: miR-21 knockout reduces hippocampal neurogenesis.

(A-D) Immunofluorescent images stained for immature neurons (DCX and Calretinin) and mature neurons (NeuN), showed reduction in neurogenesis in miR-21 KO

hippocampus, compared to WT hippocampus. Scale bar=100 µm. Highlighted box in **(C)** Representing higher magnification of NeuN staining in the DG, showed reduced volume of miR-21 KO DG compared to WT. Scale bar=10 µm. **(E)** Statistical analysis for the number of DCX showed a significant decrease in the number of DCX+ cells in the miR-21KO mice, compared to WT mice. **(F)** Statistical analysis for the number of DCX showed a significant decrease in the number of DCX+ cells in the miR-21KO mice, compared to WT mice. **(G)** A significant decrease in the number of granule cells in the DG of miR-21 KO mice compared to the WT mice. **(H)** There was a significant decrease in the volume of the DG and the CA1/3 of hippocampus of miR-21 KO mice compared to the WT mice. (n=6, mean ± SEM, $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$, one-Way ANOVA).

3.2.5 miR-21 KO mice have impaired performance in Morris water maze task

Our previous results indicated that loss of miR-21 led to decreased number of immature/mature neurons in the hippocampus, without any effects on proliferation of NPCs. To assess the impact of decreased neurogenesis in miR-21KO mice on cognitive performance, we assessed performance in a task related to learning and memory. Previous studies have linked adult neurogenesis with learning and long-term memory (Nakashiba et al. 2012; Dupret et al. 2008). Morris Water Maze (MWM) task has been linked to long-term potentiation (LTP) and NMDA receptor function in the hippocampus and is one of the common techniques to investigate learning and memory behaviours (Vorhees & Williams 2006).

Mice aged 10-12 weeks (n=9 animals from each genotype) were used in the MWM test. The testing schedule is illustrated in Figure 3-8A. The acquisition escape latency test, which is the time taken to reach a hidden platform in the MWM task, was measured across different animals. The results revealed that during days 1-5, there were significant effects for genotype, as miR-21 KO animals took significantly more time to reach the hidden platform compared to WT animals ($F_{(2-120)}$=43.46, $p<0.0001$, Figure 3-8B) over time ($F_{(4-120)}$ =9.46, $p<0.0001$). This indicates that there is a difference in the learning abilities of the mice (WT Vs miR-21 KO), whereas the second implies that mice could learn the location of the platform over the time-course of the task (days). There was no significant interaction between genotype and time ($F_{(8-120)}$ =1.23, $p<0.2885$). After five days of acquisition trials, a probe trial was conducted to measure memory consolidation. We found significant difference between groups in the time spent in the quadrant

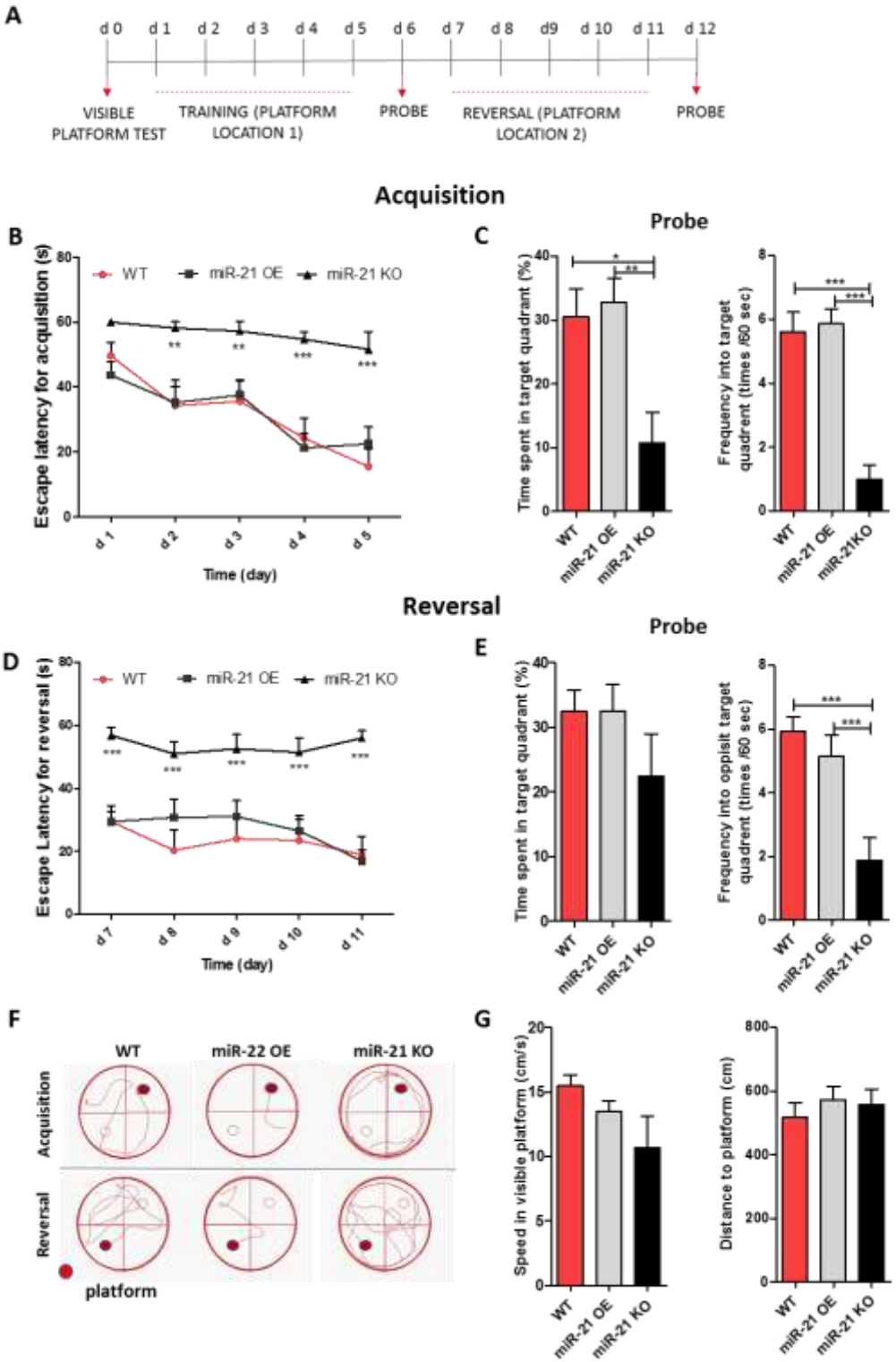
that contained the hidden platform previously. miR-21 KO mice spent less time in the quadrant that originally contained the platform compared to WT mice ($F_{(2-35)}$=7.009, p=0.0029, Figure 3-8C), and the frequency that the miR KO mice into the target quadrant was decreased compared to WT mice (Figure 3-8C). This suggesting that memory consolidation was impaired in these mice.

A reversal task was performed during days 7-11 where the hidden platform was placed in the opposite quadrant. We found a significant effect of genotype ($F_{(2-24)}$=17.38, p<0.0001) as miR-21 KO mice spent a longer time finding the hidden platform compared to WT mice (Figure 3-8D), again suggesting cognitive impairments in the miR-21 KO mice. However, the ability to identify the location of the hidden platform was improved over time (days), although results failed to reach significance ($F_{(4-96)}$=2.35, p=0.059). Therefore, although there was a difference between the ability of the mice to learn the platform location (WT vs miR-21 KO), the mice were not completely able to extinguish the memory of the previous platform location and improve latencies for days 7-11. The interaction between genotype and time ($F_{(8, 96)}$ = 1.50, p= 0.112) was, again, not significant. Reversal probe trials showed a reduction in the time spent in the quadrant that used to have the hidden platform for the miR-21 KO animals compared to WT animals, although results failed to reach significance ($F_{(2,15)}$=1.248, p=0.2543, Figure 3-8). However, when frequency to enter the target quadrant was measured, a significant difference was found between groups ($F_{(2-33)}$=10.13, p=0.0004).

The observed impairments were not due to other factors that influence these behaviors, for example visual ability, motor function and motivation. Prior to the

behavioural assessments described above, we conducted a visual platform test to confirm that the general motor abilities of the mice were not affected by their genotype. In this test, mice are motivated to swim to reach the visible platform to escape water. Thus, this test can give us an indication of the swimming ability and motivation of mice from different groups. Results showed no differences in all participating animals' swimming and visual abilities as animals from different transgenic groups had similar performance compared to WT animals in the visual platform test ($F_{(2\text{-}15)}$ =1.956, p=0.1759, Figure 3-8G). This indicates that the transgenic animals did not have any visual and motor differences compared to WT mice. Additionally, the distance covered by the animals from starting point to the platform was measured and the result showed no differences between the transgenic animals miR-21 OE and KO compared to WT ($F_{(2\text{-}33)}$ =0.4466, p=0.6436, Figure 3-8G), indicating comparable motor skills for all groups.

Taken together these results suggest that loss of miR-21 may impair learning and memory abilities and may have a negative effect on cognition.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VISIBLE PLATFORM TEST
TRAINING (PLATFORM LOCATION 1)
PROBE
REVERSAL (PLATFORM LOCATION 2)
PROBE
Acquisition
B
WT
miR-21 OE
miR-21 KO
Escape latency for acquisition (s)
Time (day)
C
Probe
Time spent in target quadrant (%)
Frequency into target quadrent (times /60 sec)
WT
miR-21 OE
miR-21KO
Reversal
D
Escape Latency for reversal (s)
Time (day)
E
Probe
Time spent in target quadrant (%)
Frequency into oppisit target quadrent (times /60 sec)
F
WT
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miR-21 KO
Acquisition
Reversal
platform
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Speed in visible platform (cm/s)
Distance to platform (cm)

Figure 3-8: Spatial acquisition and reversal task performance in Morris water maze (MWM).

(A) Experimental days outline for the Morris water maze testing showing acquisition task (day 1- day 5), reversal task (day 7-day 11) and probe trials for the acquisition and reversal tasks (days 6 and 12). **(B)** Shows the escape latency – the time mice need to reach the platform in the spatial acquisition task, in the acquisition task. **(C)** Probe trials showing the percentage of time spent in the target quadrant was increased in the WT group compared to the KO group, and the frequency into the target quadrant in the acquisition task. **(D)** Shows the escape latency in the spatial reversal task. **(E)** Percentage of time spent in the target quadrant was increased in the WT group compared to the KO group, and measurement of frequency into the target quadrant in the reversal task. **(F)** Shows representative traces of swim paths from acquisition task (day 2) of the first trial and from reversal task (day 8) of the first trial. The red circle indicates the location of the hidden platform. **(G)** Shows the speed in the visible platform test, and distance to the platform in the first day of acquisition task. Statistical significance for the escape latency was determined using Repeated measure ANOVA and for the probe trials using One-way ANOVA, $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$ (n=9, mean ± SEM).

3.2.6 MiR-21 knockdown increases the expression of the apoptotic marker Cleaved Caspase-3

During adult neurogenesis, microglia play important roles in the hippocampal neurogenic niche to regulate apoptosis of newborn cells, which occurs during the transition from late amplifying neuroprogenitors (ANPs) to neuroblasts (Sierra et al. 2010). Because miR-21 had no effects on proliferation, it was hypothesised that overexpressing miR-21 would have an anti-apoptotic effect and that knocking down miR-21 will have a pro-apoptotic effect, which has already been proposed by several studies (Hu et al. 2013b; Buscaglia & Li 2011a; Sheedy 2015; Mao et al. 2017). The release of cytochrome c from the mitochondrial membrane into the cytosol results in the activation of caspase cascades, in particular, caspase-9, -3, -6, and -7 (Gown & Willingham 2002). Inhibition of miR-21 leads to increased expression of Caspase-3, and subsequently increased apoptosis (Chan et al. 2005a; Shi et al. 2010). Analysis of Caspase-3+ staining in mouse brains of all 3 genotypes revealed that the number of Caspase-3 + cells was significantly increased in miR-21 KO hippocampus compared to miR-21 OE animals ($F_{(2-6)}$= 115.1, $p<0.0001$, Figure 3-9A), however they were not statistically different from WT mice. However more intense Caspase-3 staining was observed in the miR-21 KO mice compared to the WT mice ($F_{(2-6)}$= 6.235, $p=0.0343$, Figure 3-9). As the immunohistochemistry technique is unable to quantify protein expression levels, Western blot was performed (Figure 3-9C). Optical density (OD) values obtained for each group of mice revealed increased expression of Caspase-3 in the miR-21 KO hippocampus compared to the WT group, although results failed to reach significance (Figure 3-9D) ($F_{(2,6)}$=4.691, $p=0.0594$). Interestingly, there was an

increased expression in Caspase-3 in miR-21 OE compared to the WT group (although not reaching significant), and this was correlated with a significant decrease in the number of Caspase-3+ cells in the hippocampus compared to WT hippocampus ($F_{(2-6)}$= 115.1, p<0.0001, Figure 3-9B).

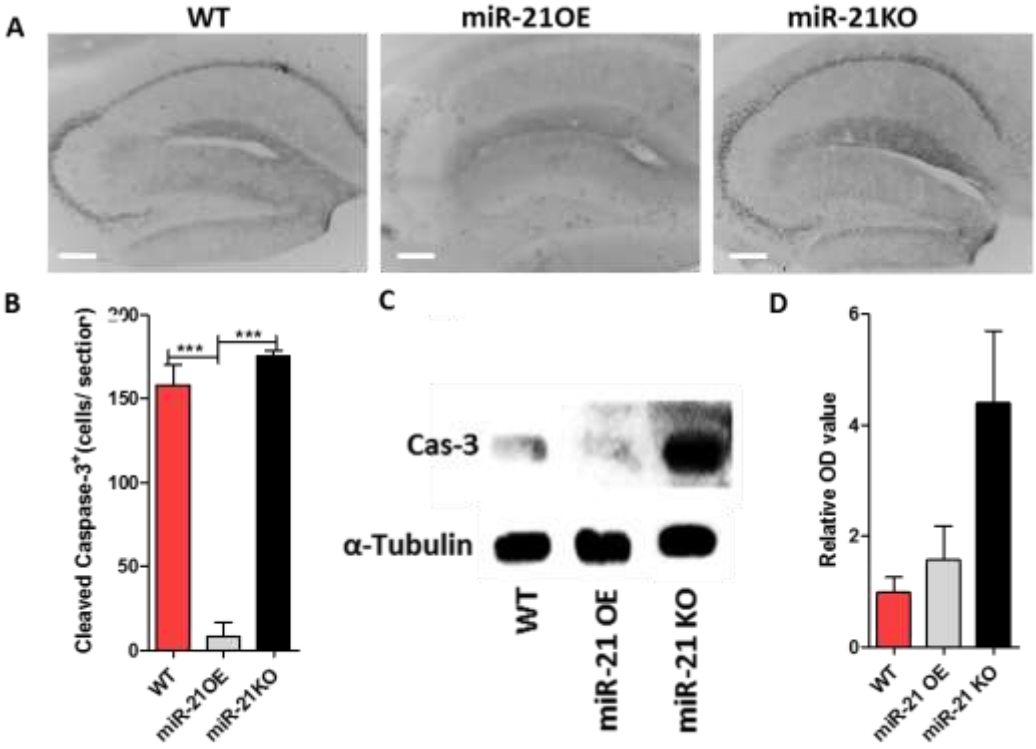


Figure 3-9: Inhibition of miR-21 mediated apoptosis.

(A) Immunohistochemistry of Caspase-3 staining for miR-21 OE, miR-21 KO compared to WT, shows that the DG cells undergo apoptosis in miR-21 KO mice. Scale bar= 100 µm. **(B)** The number of Caspase-3+ cells was decreased in miR-21 OE mice, compared to WT and miR-21 KO mice. More intense Caspase-3 staining was observed in the miR-21 KO mice. **(C)** Immunoblot showing Caspase-3 (C-C-3) and α-tubulin in hippocampal lysates. **(D)** Shows the relative optical density (OD) values obtained for each group of mice. There was increased Caspase-3 expression in miR-21 KO hippocampal lysate. The OD values for Caspase-3 were normalised to the values of α-tubulin. n=3, mean ± SEM. ***p <0.0001, one-way ANOVA.

3.2.7 Neurogenesis in P30 miR-21 KO animals is not impaired

As the transgenic mice were generated with a global knockout of miR-21 (miR-21 KO) and a global overexpression (miR-21 OE), there is a possibility that developmental compensation might have occurred. Therefore, we investigated neurogenesis at earlier developmental stages - mice at postnatal day 30 (P30) - to identify whether the above observations in the adult animals are due to developmental effect associated with earlier and/or prenatal developmental stages. Detection and quantification of immature neurons using DCX and mature neurons using NeuN showed no differences in the number of DCX+ or NeuN+ cells in the miR-21 OE and miR-21 KO brains compared to the WT animals (Figure 3-10A, C). There was also no significant difference in the hippocampal volume of the miR-21 OE and miR-21 KO compared to WT mice, as assessed by NeuN staining (Figure 3-10B, D). This indicates that the results obtained in this study are due to miR-21 effects in the adult stage and not due to developmental effects related to altered miR-21 expression.

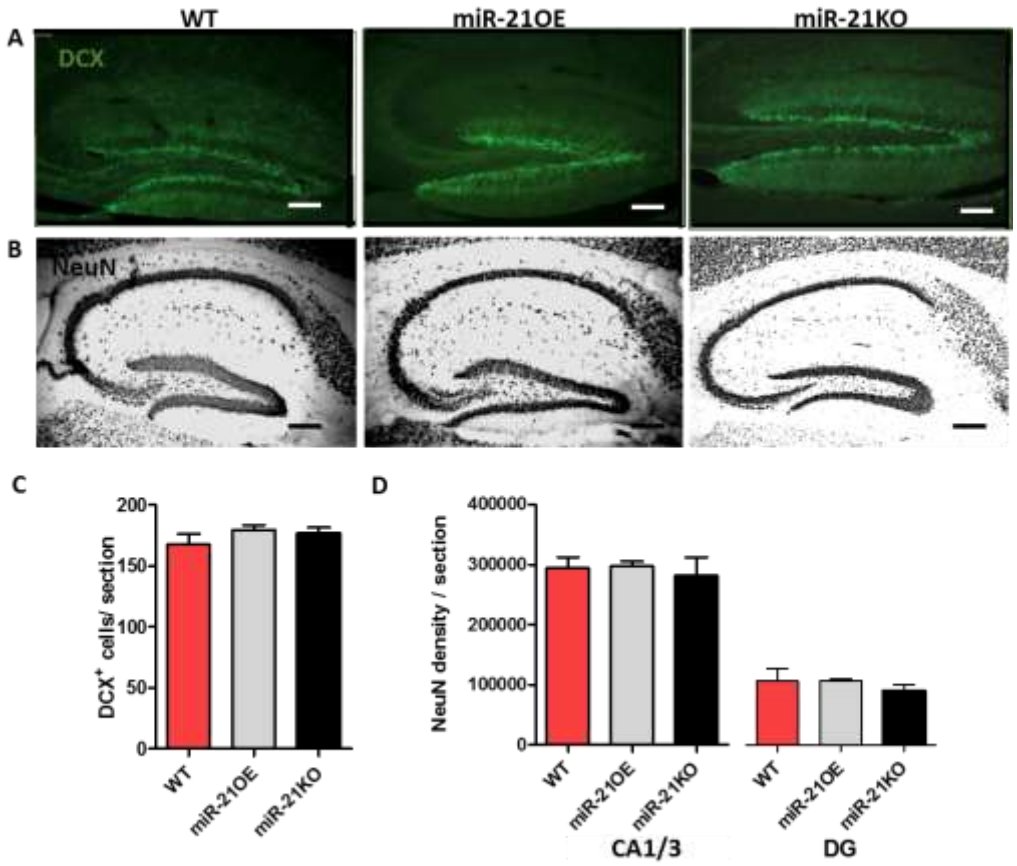


Figure 3-10: Neurogenesis is not altered in miR-21 KO animals at P30.

(A-B) Detection of immature DCX and mature NeuN neurons in the hippocampus. Scale bar=100µm. **(C-D)** Quantification of DCX+ cells and DG/hippocampal volume in transgenic P30 mice. The number of DCX+ cells and hippocampal volume did not differ between groups. n=3, mean ± SEM. One-way ANOVA.

3.2.8 Improved adult neurogenesis in aged OE mice

NPCs have the ability to proliferate throughout life in the SGZ and the SVZ to contribute to brain function such as learning, memory and repair. However, a marked reduction in the proliferation rate of NPC is associated with age, as the adult neurogenesis rate in mice declines after the early adult stage (2 months) to reach its minimum in mice aged 12-24 months (Nunez-Parra et al. 2011). Identifying factors involved in the maintenance of adult neurogenesis can provide a therapeutic tool to be used to improve brain functions and to hinder or prevent neurodegenerative diseases. Over expression of miR-21 resulted in increased immature (DCX) and mature (NeuN) neuronal production, compared to WT animals (Figure 3-7). As these observations were made in adult mice aged 3-4 months, we wanted to investigate whether this increase was maintained in aged animals (Figure 3-11). Analysis of brains from aged (12 months) miR-21 OE, miR-21 KO mice showed that DCX+ cells in the miR-21 OE animals were significantly higher than in the miR-21 KO mice ($F_{(2,6)}$= 197.8, $p < 0.0001$). However, DCX+ cell number was reduced in miR-21 OE compared to WT ($F_{(2,6)}$= 197.8, $p < 0.001$), indicating that the effects of miR-21 were not maintained beyond the adult stage of development.

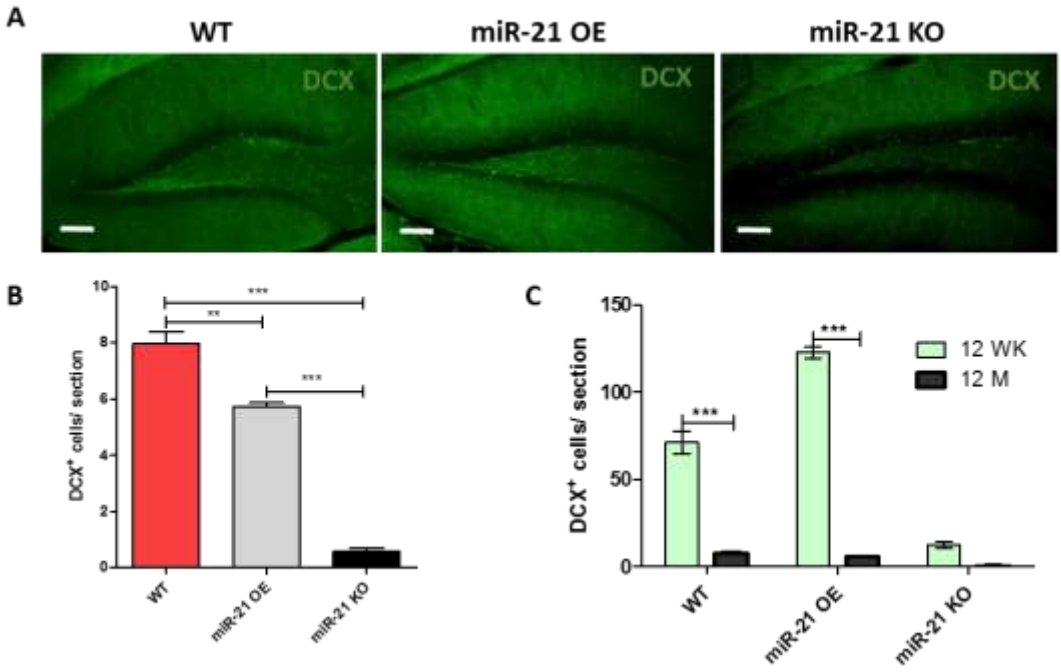


Figure 3-11: Impaired neurogenesis in aged (12 months) mice.

(A) Representative images showing immature (DCX) neurons in miR-21 OE and KO mice sections compared to WT. Scale bar=100μm. **(B)** Quantification of DCX+ cells shows significant reduction in the number of DCX+ cells in miR-21 KO and WT. **(C)** Neurogenesis was reduced significantly in older mice. n=3, mean ± SEM. One-way ANOVA.

3.3 Discussion

In the brain, new neurons are continuously generated from neural stem cells in the SGZ and the SVZ throughout adulthood and contribute to memory processing (Aimone et al. 2014; Aimone et al. 2010; Pignatelli & Belluzzi 2010; Altman & Das 1965; Deng et al. 2010). Identification of specific molecules that are involved in the regulation of adult neurogenesis can contribute to understanding underlying neurogenesis mechanisms and related pathogenesis. miR-21 is a miRNA that is known for its pro-proliferative role in the progression of many cancer types as well as improved recovery after TBI and SCI (Redell et al. 2011; Sheedy 2015; Selcuklu et al. 2009). The upregulation of miR-21 coincides with the regulation of multiple genes associated with tumour suppression activities, cell proliferation, development and survival (Sandhir et al. 2014; Ge et al. 2014; Strickland et al. 2011). In addition, previous studies have found that the total lack of miR-21 in the aged brain contributes to poor outcomes after controlled cortical impact injury, a form of TBI (Sandhir et al. 2014). Therefore, we speculated on the possible involvement of miR-21 in adult neurogenesis.

Here we investigated if the loss of miR-21 would affect proliferation and neurogenesis primarily in the SGZ. To decipher the role of miR-21, we used transgenic mice (CAG- miR21-EGFP) that overexpressed the miR-21 gene miR-21 OE and mice that were deficient in miR-21 (miR-21 KO) (Hatley et al. 2010). We validated the level of miR-21 expression in the transgenic animals by q-PCR and found, as expected, that miR-21 OE mice have a 4-6 fold increase in miR-21 expression, and miR-21 was absent in miR-21 KO mice. However, the most important finding in our study is that loss of miR-21 results in a significant

reduction in adult neurogenesis, which resulted in impaired spatial learning performance in the MWM task. The impaired neurogenesis in the miR-21 KO mice is likely due to the regulation of apoptosis.

Põlajeva et al. (2012) found that miR-21 is highly expressed at embryogenesis stages (E18) and earlier developmental stages (P1). However, miR-21 expression was strongly reduced at later developmental stages (P7). They found that miR-21 is co-expressed with SOX2, which is required to maintain cells pluripotency during embryogenesis (Põlajeva et al. 2012). However, several studies have shown increased miR-21 expression in diseased brains, for example after traumatic injury (Redell et al. 2011), after stroke (Buller et al. 2010) and in neurodegenerative diseases (Montalban et al. 2014). Additionally, the expression of miR-21 is co-localised with neurons, astrocytes, oligodendrocytes and microglia in the adult brain (Ge et al. 2014; Miguel-Hidalgo et al. 2017; Simeoli et al. 2017). Others have shown low expression of miR-21 in normal adult brain compared to elevated miR-21 expression after stroke (Buller et al. 2010), indicating that expression of miR-21 can be stimulated in pathological conditions.

Our results indicated that miR-21 is expressed in the adult brain, mostly in the hippocampus and in the SVZ and corpus collosum (Figure 3-4). However, our attempts to localise miR-21 with different cell types were not completely successful (Figure 3-5). We were able to detect miR-21 in astrocytes but were unable to confirm its presence in neurons or microglia, therefore further investigations with optimized protocols will be required. To overcome the failure of the FISH experiment, other methods can be applied to enable fluorescence

labelling of miR-21 such as the use of a lentiviral construct of miR-21 tracer system, which enable the labelling of cells that express miR-21, as the tracer express green fluorescence protein (GFP) constitutively, but express red fluorescence protein (RFP) only when miR-21 is not expressed (Han et al. 2016). Recently, it has been suggested that miRNAs are secreted by exosomes, which are small vesicles (30-150 nm) released by different types of cells, including neurons (Cicero et al. 2015). These secreted exosomes contain a specific cargo composition including miRNAs to regulate neural functions (Chivet et al. 2012). We speculate that exosomes containing miR-21 are released in an activity-dependent manner to regulate adult neurogenesis.

Nonetheless, it is clear that miR-21 is expressed in the adult brain, as evidenced by qPCR data and limited success of the FISH. We next wanted to investigate the correlation between miR-21 expression and hippocampal adult neurogenesis. We first studied the impact of miR-21 ablation on the neural populations in the DG by using markers of proliferation (BrdU, Ki67), neurons (DCX, CR, NeuN) and astrocytes (GFAP). We showed that neither the loss or gain of miR-21 affected NPC proliferation, a finding that differed from the miR-21 function in stimulating cell proliferation in many cancer types (Yu et al. 2012; Selcuklu et al. 2009a). This inconsistency can be explained by the proposition that different miRNAs perform different functions under specific conditions or context (Flynt & Lai 2008). miRNA can be associated with different groups of target genes under different conditions, thus identifying the genes targeted by miRNAs in a given context is important (Oh et al. 2017).

Adult hippocampal neurogenesis comprises multiple steps, including the proliferation of neural progenitors, differentiation to neurons, and survival of these newborn neurons. Although no change was detected in the rate of proliferation, the loss of miR-21 was sufficient to cause reduced neurogenesis, as evidenced by decreased number of cells that were positive for the DCX, Calretinin and NeuN markers. This suggested that miR-21 affects the survival of immature neurons, or the differentiation potential of progenitors. Quantification of the number of astrocytes showed no differences in the different groups of mice. However, to improve on the quality of this data, we may need to perform experiments where cells labeled with BrdU+/GFAP+ along with cells with unknown phenotype BrdU+/ NeuN-/ GFAP- are quantified.

The observed reduction in hippocampal neurogenesis in miR-21 KO and enhanced hippocampal neurogenesis in miR-21 OE mice compared to the WT mice, led us to study their learning and memory abilities using the Morris water maze test. We found that the reduced neurogenesis in miR-21 KO mice resulted in impairment in cognitive performance. Despite similar performances amongst all genotypes in terms of vision and motor activity (Figure 3-8G), we found that the KO mice exhibited reduced memory acquisition and retention compared to the WT animals. Speed is an important factor in the escape latency test, as reduced speed may result in a longer time taken to reach the platform, and this indicates impaired motor functions but not cognitive abilities. Therefore, the speeds of the different animals were analysed in a visible platform test, and we found that all animals had similar speeds. However, when traces of swim paths were analysed, it was found that some

of the KO animals had thigmotaxis, meaning that the animals stayed almost exclusively in the periphery of the arena, repeatedly searching for contact near walls. One of the things that experimental animals need to learn in the water maze is that there is no escape located around the edges of the tank and most animals learn to swim away from the wall to find the escape which is the platform. Animals that fail to learn is an indication that the animal does not have adequate awareness of its surroundings (Vorhees & Williams 2006). Excessive thigmotaxis (especially in rats) indicates that the animal is not focusing on the task appropriately (Treit & Fundytus, 1988). Also, it was noticeable that a number of miR-21 KO animals showed excessive jump-off behaviour when they reached the platform but did not stay on it. This behaviour may indicate that animals are not acquiring the association between the platform and escape. These sorts of behaviours are acceptable in the first two days of the experiment, but can be an indication of impaired spatial learning if repeated during the whole experiment (Vorhees & Williams 2006). This behaviour can be explained by miR-21 KO mice lacking the ability to learn tasks and have a decreased flexibility to cope with changes, which could be a consequence of reduced neurogenesis. This can result in a lack of plasticity of the GCL of the DG, thus preventing the effectiveness of DG network adaptation. It might be that miR-21 KO animals needed extra training in non-spatial strategy sessions before the acquisition task, in which they learn the basic task requirements by covering the visual cues and learning how to reach the platform. This strategy of cued training has been suggested to eliminate behaviours such as swim-overs and jump-offs (Vorhees & Williams, 2006).

Our findings of impaired cognitive performance in the KO animals were similar to previous work that also revealed reduced performance associated with miR-21 inhibition after TBI in MWM tasks, which strengthens the idea of the role of miR-21 in enhancing cognitive performance (Ge et al. 2014; Hu et al. 2015). Despite the fact that the MWM test is one of the most commonly used tasks to test for hippocampus related memory, there are other tests that are thought to better dissect the function of adult neurogenesis in cognition. Other examples of these behavioural tests include: pattern separation, which enables the discrimination among similar experiences by using a touch screen devise (Clelland et al. 2009). In this task mice had to remember a number of visual patterns, which can be either distinct in space or close to each other for more a complicated task. Another task is the match to sample test (MTS), which is based on presenting a subject with a stimulus such as light, or colour or visual pattern that animals need to remember, and the test requiring the identification of correct stimuli. This test is similar to the delayed non match to sample test (DNMTS), but the latter is more a complicated task as it includes a longer interval time between sample and test trials (Winocur et al. 2006; Zhao et al. 2008; Bielefeld et al. 2017).

Plasticity is involved in the regulation of the process of restoring and extracting past information, which is important for adaptation especially in new and changing environment. The N-methyl-D-aspartic acid (NMDA) receptor controls synaptic plasticity and memory function, and is also associated with memory formation (Vorhees & Williams 2006). In addition, NMDA receptor was found to be involved in the regulation of synaptic changes after injury and in several

neurogenerative diseases, and involves in the stimulation of miR-21 expression (Yelamanchili et al. 2010). Identification of how the regulatory mechanisms underlying NMDA pathway is associated with miR-21 altered expression may reveal further explanation for impaired performances in miR-21 KO mice.

To identify the factors involved in reduced neurogenesis associated with miR-21 KO, we investigated Caspase-3 expression in the hippocampus of transgenic mice. miR-21 has been linked to the inhibition of cell apoptosis through the regulation of tumour suppressor and pro-apoptotic genes including *PTEN*, *SPRY1*, *SPRY2*, *PDCD4*, *Faslg*, *Apaf* and *Btg2* (Yang et al. 2014; Si et al. 2007; Hatley et al. 2010). However, the exact molecular mechanisms involved in miR-21 function in the normal brain remains unclear. Studies have shown that the activity of Caspase-3 and Caspase-9 in the miR-21-null brains is increased, supporting the role of miR-21 in inhibiting apoptotic activity (Buscaglia & Li 2011). MiR-21's link with apoptosis may be exerted through two pathways: an intrinsic mitochondrial pathway or an extrinsic pathway induced by cell surface receptors and the granzyme B pathway, although some molecules in one pathway can interact and influence the other (Buscaglia & Li 2011). Immunohistochemistry quantification analysis revealed that the number of Caspase-3+ cells was significantly increased in miR-21 KO mice compared to miR-21 OE mice, but not to WT mice. To validate this result Western blotting was performed, which suggested increased Caspase 3 expression in miR-21 KO mice, although this did not reach significance (Figure 3-9D). This result was similar to previous findings that showed that ablation of miR-21 by antisense oligonucleotides in a number of glioblastoma-cell

lines resulted in increased expression of Caspase-3 (Chan et al. 2005). Similarly overexpression of miR-21 in U87 glioma cells led to a reduction in pro-apoptotic B cell lymphoma 2 (Bcl2) expression, which are pro-survival protein (Shi et al. 2010). Although Caspase-3 is not a direct target for miR-21 (Hatley et al. 2010), it might be that miR-21 regulates Caspase-3 indirectly though the regulation of its direct target Bcl2. However, identification of other regulatory elements and binding associations of miR-21 can help to find other direct targets of miR-21 during neurogenesis. For example, a previous study showed that miR-21 expression is regulated by a protein complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which binds the miR-21 promoter and regulates its expression by the modulation of PTEN target protein, thus enhancing cell survival (Yang et al. 2015).

Microglia in the adult brain can interact with NPCs either in the DG or in the SVZ and initiate phagocytosis and eventually apoptosis. Thus, microglia can be associated with most of the granule cell death during the survival stage of neurogenesis (Sierra et al. 2010). The proper integration of adult newborn neurons with the existing neural network is necessary for new neurons to survive and associated cognitive behaviour (Kempermann et al. 2015). If this integration is impaired, it will lead to newborn cells undergoing apoptosis and then being phagocytosed by local microglia (Askew et al. 2017; Sierra et al. 2010). Microglia are resident myeloid cells of the CNS that have an important role in the maintenance of homeostasis in normal (Bennett et al. 2016). Quantification of the number of activated microglia, as an indication of phagocytosis, in the hilus of

miR-21 transgenic mice compared to the WT mice can further validate our Caspase-3 results. A qualitative analysis can indicate if there is an increase reactive microglia, based on their morphological features, related to increased phagocytosis (Yip et al. 2017). Although there is not a specific marker for reactivated microglia, a more accurate quantification of microglia can be done by immunostaining for microglia by a newly discovered marker TMEM119 (Bennett et al. 2016). TMEM119 is not expressed by macrophages and can be used in combination with Ionized calcium binding adaptor molecule 1 (Iba1) marker for all microglial types to confirm more specifically if Iba-1 positive cells are microglia.

Impaired learning and memory occurs during the aging process and is associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. There is a rapid decline in the basal hippocampal neurogenesis associated with age, and reduced neurogenesis activation after stroke and injury, due to impaired maturation and survival of NPCs in older animals (Darsalia et al. 2005; Kuhn et al. 1996a). Additionally, low levels of neurogenesis detected in aged animals can be attributed to increased inflammatory functions associated with microglia activation and increased age (Solano Fonseca et al. 2016). Thus, investigation of the role of elevated expression of miR-21 in aged animals or in animals with a neurogenerative disease is required to determine if increased expression of miR-21 will compensate for reduced neurogenesis in older animals.

The observed effects of miR-21 loss on neurogenesis and cognition is not due to developmental deficits. The evaluation of the neural progenitor and mature neuron populations at postnatal day 30 (P30) revealed no difference in the number of

immature neurons (DCX+) cells nor the density of mature neurons (NeuN) in the DG and CA3 and CA1, indicating that results obtained were specific to adult neurogenesis and not because of neurogenesis regulation during embryonic and postnatal embryonic developmental stages.

This showed that miR-21 is involved in the regulation of hippocampal adult neurogenesis *in vivo*. It will be important to identify specific types of cells expressing miR-21 in the hippocampal niche, as well as expression of other miRNAs to define the full complement of miRNAs expression in the region. This can reveal other functional activities of miR-21, in combination with other miRNAs, besides its role in adult neurogenesis. Schouten et al. (2015), for example, found that BCL-2-Like 13 (BCL2L13), which is a member of Bcl2 family, is regulated in the NPCs in the epileptic brain by the cooperative function of miR-124 and miR-137 activity. Each miRNA did not have a significant effect on BCL2L13 protein levels or Caspase-3 activation individually but exerted effects when administered together. Additionally, they postulated that the apoptotic protein Caspase-3 was involved in NPC differentiation and survival, as it is regulated by BCL2L13 (Schouten et al. 2015). Taking into account that Bcl2 is a direct target for miR-21, it is possible that a similar mechanism may operate for miR-21 regulation of adult neurogenesis.

This study found that miR-21 KO led to impaired hippocampal neurogenesis, indicating that miR-21 is involved in regulating neurogenesis in normal brain. Therefore, understanding the effects mediated by miR-21 can help to uncover new factors to stimulate neurogenesis in non-neurogenic brain areas. Several studies

pointed the role of miR-21 in regulating apoptosis. For example, a study by Biswas et al. (2017) investigated mechanisms involved in cell apoptosis in Alzheimer's disease (AD), found miR-21 involvement in the apoptotic cell cycle pathway in normal and AD brain. Stimulation of neural death induced by deprivation of nerve growth factor (NGF) and Aβ resulted in increased expression cell division cycle 25A (Cdc25), which is regulated by transcription factor Forkhead box O3 (FoxO3a) that suppress miR-21, a negative regulator or Cdc25. Elevated Cdc25 expression increased Caspase activity and thus increased neuronal cell death (Chatterjee et al. 2016). Taken together, further investigation of miR-21 direct targets and their role normal and diseased brain may serve as a tool to protect from neural death in neurodegenerative diseases.

Finally, the finding of this study can be used to further investigations into the role of miR-21 in hippocampal neurogenesis. Additionally, enhanced neurogenesis associated increased expression of miR-21 can be used as a therapeutic target to reduce cell apoptosis in neurodegenerative diseases or after brain injury.

Chapter 4 miR-21 target prediction and validation

4.1 Introduction

miRNAs are small (18-22 nt) non-coding RNAs that are involved in post transcriptional regulation in plants and animals. Nearly all miRNAs act to repress their target mRNAs through imperfect binding to the 3' untranslated region (3'UTR) of mRNAs to inhibit translation or degradation. The coding region of mRNAs includes miRNA recognition elements (MREs), and the most important factor in miRNA target recognition is the nucleotides at position 2-8 (seed region). The loose complementarity of miRNAs in terms of binding to their targeted mRNAs results in multiple targets for each miRNA. Additionally, multiple miRNAs can target the same mRNA, leading to a complex regulation network. It has been estimated that over 60% of the human genome is under the regulation of miRNAs (Catalanotto et al. 2016). Since the discovery of miRNAs, they have been recognised to be involved in the regulation of gene expression in various cellular activities in many homoeostatic processes and pathological conditions, including development, differentiation, metabolism, induction of apoptosis and disease pathology. The overexpression/downregulation of miRNA will dysregulate target genes and this has been observed in the pathophysiology process of disease. For example, downregulation of Let-7 results in the development of lung cancer (Takamizawa et al. 2004); miR-21 overexpression is linked with cardiovascular diseases (Cheng & Zhang 2010) and overexpression of miR-155 inhibits apoptosis in leukaemia patients (Palma et al. 2014), to name but a few examples. Moreover, miRNAs have been associated with the development of different cancer types and their progression. Oncogenic miRNAs (oncomiRs) can regulate genes that have been associated with tumour suppressor functions, through the maintenance of cell

proliferation and survival (Lin & Gregory 2015). Therefore, the functions of miRNAs in normal and diseased tissues have been studied to understand their role and assist in the development of new drugs and therapeutic strategies (Ling et al. 2013).

However, the investigation of the role of miRNAs in the regulation of their target mRNAs can be complicated. This is due to the small size of miRNAs, which requires 6-8 base pairs of seed region complementarity for effective miRNA targeting. However, as complete complementarity is not essential for miRNAs to exert their effects, the task of accurately determining 'true' miRNA targets with high binding specificity is challenging.

In addition, the dynamics of the crosstalk between mRNAs and miRNAs add another layer of complication. One miRNA can interact with two mRNAs and *vice versa*, as well as a group of miRNAs can interact with one or more mRNAs and *vice versa* (Figure 4-1). The resultant effect indicates that multiple targets can be targeted by one miRNA and one miRNA can be regulated by several targets (Peter 2010).

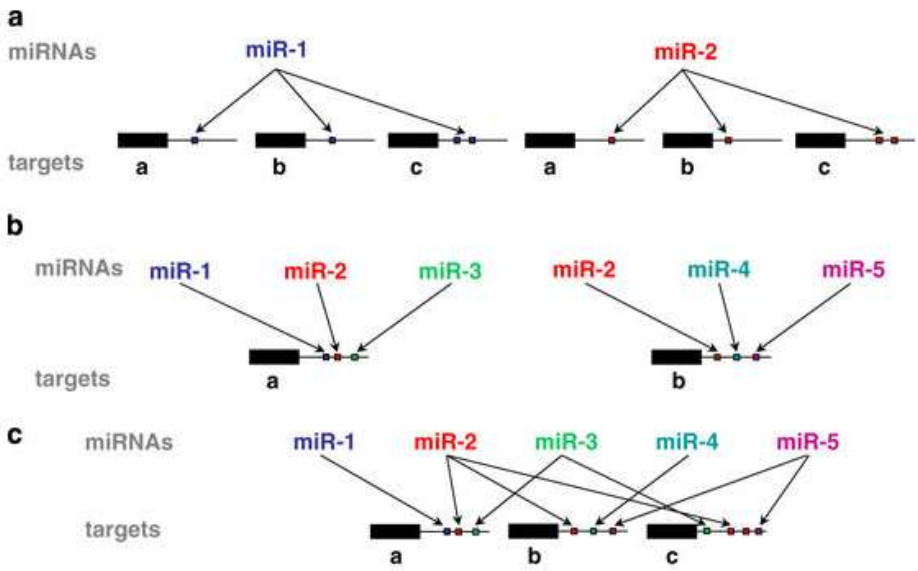


Figure 4-1: microRNA-mediated gene regulatory networks through dynamics of the crosstalk.

(a) One miRNA can regulate the function of multiple genes. **(b)** Many genes have seed matches in their 3'UTR for multiple miRNAs. **(c)** A complex interaction network between multiple miRNAs can regulate the function of a group of genes as more than one miRNA can regulate the function of one gene. The image is taken from (Peter 2010).

The development of next-generation sequencing (NGS) has led to an increase in the amount of available data on miRNA. To better store and annotate this data, new bioinformatics approaches have been developed. Additionally, analysis of these sequencing data has provided better understanding of miRNA functions. Subsequently, different computational prediction programs have been created to identify mRNAs that are targets of specific miRNAs. Computational algorithms have been developed based on observed rules for features such as the degree of hybridization, degree of sequence complementarity, free energy of binding and site accessibility between the two RNA molecules to enable the identification of a possible miRNA target.

4.1.1 Principles of miRNA target prediction

Most of the computational databases identify complementary miRNA-mRNA conserved sequences and calculate the thermodynamics between the duplex. However, each algorithm allocates different weighting or importance across a number of factors and therefore individual bioinformatic analyses can result in a different set of predicted mRNA targets. The principles of miRNA target prediction include: seed sequence complementarity, conservation, thermodynamics and site accessibility.

4.1.1.1 Seed sequence complementarity

Seed sequence complementarity is considered the most important criterion in all prediction algorithm tools. It takes into consideration the imperfect matches between the 3'UTR in the mRNA and the mature miRNA, with particular attention to the seed region in the miRNA. Binding occurs at the 5' end of the mature miRNA to the 3'UTR mRNA sequence. There are three types of target sites: 5′-dominant canonical, 5′-dominant seed only and 3′-compensatory (Figure 4-2). These differ in terms of the level of miRNA complementarity to the target site sequences along the length of the miRNA sequence. To increase the signal-to-noise ratio, some methods require strict complementarity between the seed region of the miRNA and the predicted target. However, non-canonical target sites with imperfect seed complementarity with miRNA have also been reported to be functional; and this may bring the specificity of the databases into question (Lewis et al. 2003; Seok et al. 2016; Brodersen & Voinnet 2009).

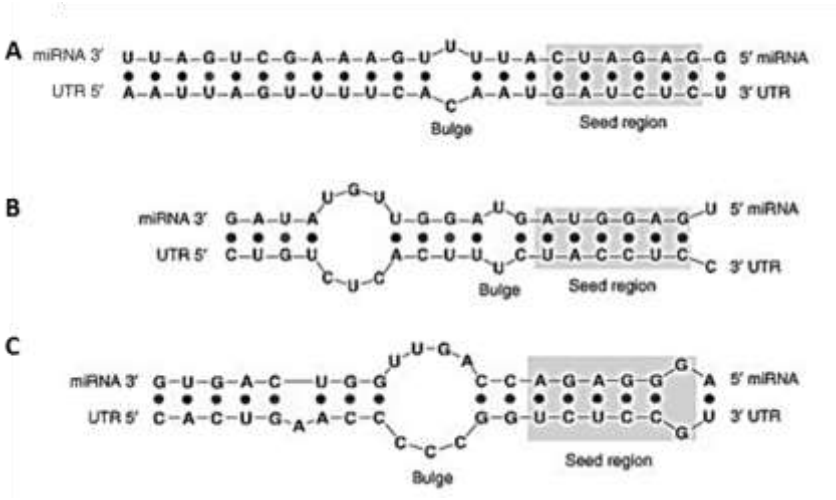


Figure 4-2: Representative diagram showing the three main types of target site duplex.

(A) Canonical sites with good to perfect complementarity at both ends, the 5′ and 3′ of the miRNA. **(B)** Dominant seed sites have perfect 5′ complementarity but poor 3′ complementarity with the miRNA. **(C)** Compensatory sites have a mismatch or wobble at the 5′ end region but good to perfect complementarity at the 3′ end of miRNA. Image taken from (Mazière & Enright 2007).

4.1.1.2 Conservation

Many prediction tools take into account conservation of the miRNA binding sequence to the mRNA target across different species. A highly conserved target site is considered a more reliable predictor for the target gene because an important miRNA-mRNA interaction is more likely to be evolutionarily conserved over time. The comparative study of miRNA seed regions, and their 3'UTR mRNA target in different species, is used to reduce the number of false positives. Many target prediction algorithms select orthologous 3'UTR sequences and perform a conservation test among different species to improve target detection specificity. For example, the evolutionary conservation pattern of different species including zebrafish, chicken, opossum, rat, mouse, cow, dog, and chimpanzee etc. is

examined to detect for conserved sequences across various species. In miR-21-5p, the 5´-AUAAGCUA-3´ sequences were highly conserved within the 3' UTR of its target gene *Pdcd4* from the lizard to primates (Figure 4-3). However, conservation alone is not sufficient for measuring the strength of a site and should be used in combination with other features for target site scoring. Some functional targets are loosely conserved at specific positions and previous reports have suggested that using conservation as a filter led to a substantial number of downregulated targets being missed, which may result in increasing the number of false negatives (Hammell et al. 2008; Betel et al. 2010)

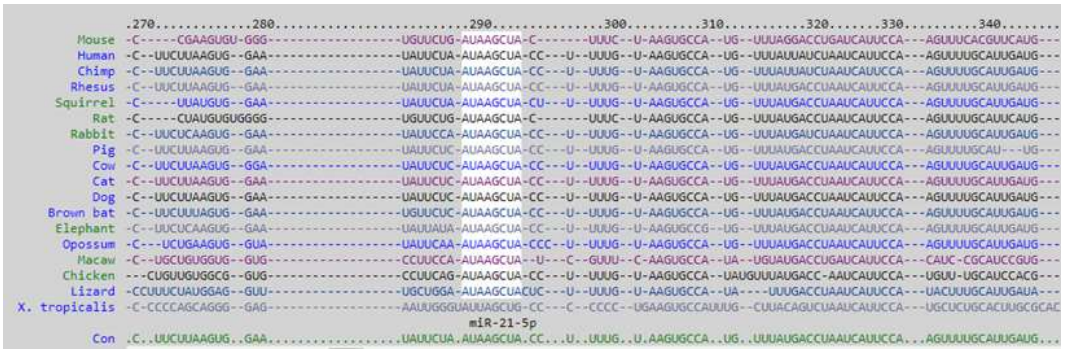


Figure 4-3: Comparative analysis of miR-21-5p in different species.

The white region 5'-AUAAGCUA-3' was highly conserved in the 3'UTR of PDCD4 mRNA in different species. Picture taken from miRBase (http://www.mirbase.org).

4.1.1.3 Low thermodynamics

Thermodynamics is the calculation of duplex free-energy (ΔG) upon binding of the miRNA to the mRNA. During the binding reaction, free energy between the miRNA and its target candidate changes. Hybridisation of the miRNA and mRNA results in a more stable and energetically preferable state, and their free energy is

low, which means the paired strands need more energy to be disrupted and will have less energy available for future reactions (Figure 4-4) (Lewis et al., 2005a). Thus, free energy Δ*G* can be used as an indicator of strength and stability of binding (Yue et al., 2009). For example the minimum free energies (Δ*G*) for binding of miR-21 to PDCD4 3'-UTR is −2.69 kcal/mol (Zhang et al. 2016), to PTEN 3′-UTR is −13.7 kcal/mol (Dey et al. 2011), and to TGFBR2 3′-UTR is −52.4 kcal/mol (Chak et al. 2016).

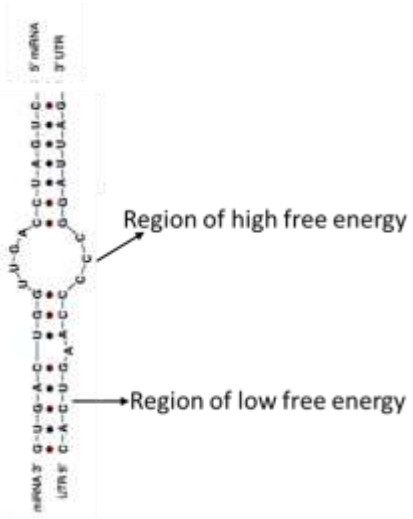


Figure 4-4: Schematic diagram representing an overview of free energy (ΔG) analysis of miRNA: mRNA hybridisation structure.

A bulge hairpin loop represents a region of high free energy and the stem represents a region of low free energy.

4.1.1.4 Site accessibility

To enable the binding of the miRNA to the target mRNA, the secondary structure of the mRNA has to be made accessible to the miRNA. Site accessibility is the measurement of the ease with which the miRNA locates and hybridises with its

allocated target. Following transcription mRNA molecules can form secondary structures that can reduce the ability of miRNA to bind to the complementary mRNA site. Embedding of the mRNA 3‘UTR in a secondary structure may reduce the accessibility of the putative target sites to miRNAs irrespective of the sequence complementarity and interaction free energy. Hybridisation between miRNA and mRNA requires the binding of miRNA to a short region in the mRNA, which encourages the unfolding of the mRNA secondary structure to complete the binding (Long et al. 2007). To encourage the binding, an additional short region (~15 nt) upstream and downstream of the target site has to be opened as well. Therefore, the amount of energy required to make an mRNA site accessible to a miRNA can be predicted and evaluated.

4.1.2 miRNA target prediction tools

Since the discovery of miRNAs, many computational approaches have been developed to enable the identification of possible targets. These tools use different feature combinations, making it hard to decide which of these tools produce reliable results. A comparison between all different computational predictions on microRNA.org has identified reasonable similarities between a number of these databases such as Target scan (http://www.targetscan.org/vert_72/) (Agarwal et al. 2015), miRDB (http://www.mirdb.org/) (Wong & Wang 2015b), Pictar (https://pictar.mdc-berlin.de/) (Krek et al. 2005) and DIANA-microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) (Paraskevopoulou et al. 2013).

TargetScan (http://www.targetscan.org/vert_72/) is one of the tools that detects 3'UTR targets by perfect seed matching complementarity of a miRNA (Agarwal et al. 2015). Additionally, the database uses a method of detection that extends the matching region to find complementarity outside the seed region. This results in reducing many false positives from the prediction process. Furthermore, the tool searches for both evolutionary conserved and non-conserved target sites by comparing between orthologous 3'UTRs from different species. Each of the identified targets is given a score based on their conservation across species and the AU composition of the flanking region. This tool is updated regularly with the aid of the laboratory of David Bartel. The database addresses the predicted binding sites' thermodynamic stability using RNA Fold from the Vienna Package (Mazière & Enright 2007). It has been estimated that TargetScan has a high specificity with the estimated false-positive rate to be between 22% and 31%.

miRDB (http://mirdb.org/) is another target prediction tool, which is a combination of two databases; computationally predicted miRNA targets and miRNA functional catalogue (Wang 2008). The tool target prediction algorithm has been improved to increase prediction sensitivity and specificity. The scoring method relies on four criteria, which are then calculated to identify the level of miRNA functionality. The first criterion is based on the number of PubMed publications, given a score from 1 to 4. The second is the conservation score depending on the availability of orthologous miRNA in multiple species including human, mouse, rat, dog and chicken. The third criterion is based on the normalized read counts for miRNA expression from RNA-seq experiments. The last criterion is the sum of all

of the three criteria. Thus, a score more than 3 can indicates functional miRNA. This tool has 2.1 million predicted gene targets regulated by 6709 miRNAs, which were all predicted by the MirTarget (miRDB) bioinformatics database (Wong & Wang 2015). Further analysis of miRDB in relation to other prediction algorithms including TargetScan, PicTar and miRanda indicated better prediction results associated with miRDB and TargetScan compared to PicTar or miRanda (Wang & El Naqa 2008).

DIANA-microT-CDS (www.microrna.gr) is part of a DIANA web-based tool that provides access to different tools for miRNA analysis and was first developed in 2004 (Kiriakidou et al. 2004). The tool has two main parameters to detect possible targets; the first is the calculation of the free energy between the miRNA and the putative 3'UTR of mRNA. The second parameter relies on the size and the position of the loops and the hybridised nucleotides of miRNA and the targeted mRNA. This tool was updated to include more features such as target prediction score and KEGG description with associated pathways. Additionally, the tool uses miRNA binding site with the conservation profile of up to 27 species to evaluate each miRNA:target interaction, which can reduce background noise. The updated version of DIANA-microT-CDS has included more species including human, mouse, fly and worm species, and the prediction algorithm has been further developed. More importantly, the tool has incorporated different parameters to compare prediction results with other bioinformatics tools (Paraskevopoulou et al. 2013). DIANA-microT-CDS website allows the identification of experimentally validated miRNA targets on coding and non-coding RNAs (Mazière & Enright

2007). A summary of the available computational tools for predicting target mRNAs of specific miRNAs are illustrated in Table 4-1.

Tool name	Seed match	Conservation	Free energy	Site accessibility	Target-site abundance	Machine learning	References
miRanda	X	X	X				Enright et al., 2003; John et al., 2004
miRanda-mirSVR	X	X	X	X		X	Betel et al., 2010
TargetScan	X	X					Lewis et al., 2005; Grimson et al., 2007; Friedman et al., 2009; Garcia et al., 2011
DIANA-microT-CDS	X	X	X	X	X	X	Maragkakis et al., 2009; Reczko et al., 2012; Paraskevopoulou et al., 2013
MirTarget2	X	X	X	X		X	Wang, 2008; Wang and El Naqa, 2008
RNA22-GUI	X		X				Hofacker et al., 1994; Miranda et al., 2006; Loher and Rigoutsos, 2012
TargetMiner	X	X	X	X	X	X	Bandyopadhyay and Mitra, 2009
SVMicrO	X	X	X	X	X	X	Liu et al., 2010
PITA	X	X	X	X	X		Kertesz et al., 2007
RNAhybrid	X		X		X		Rehmsmeier et al., 2004; Kruger and Rehmsmeier, 2006

Table 4-1: Summary of some the target predicted tools and their features.

The table is adopted from Peterson et al. (2014).

4.1.3 Experimental validation of predicted targets

Based on bioinformatics analysis, over 2000 known miRNAs are targeting up to 30% of protein-coding genes in the human genome (Lewis et al. 2005). However, the false positive rate of prediction programs can be up to 70%, which begs the question of the accuracy of these bioinformatics tools. Additionally, investigation of miRNA targets using different computational tools may result in different interaction lists leading to confusion. To date there is no uniform algorithm that can be used for all miRNA identification analysis. For example, even perfect seed pairing is not a reliable target predictor for miRNA-target interactions as some validated miRNA target sites do not have a complete seed match (Didiano & Hobert 2006). Therefore, reliable identification of miRNA:mRNA interactions will need experimental validation to prove true functionality. Additionally, experimental validation is important in order to elucidate miRNA functions in different conditions and species. This emphasizes the need for experimental data to validate genuine miRNA targets and associated miRNA function (Witkos et al. 2011).

In order for the miRNA to repress the expression of its related target, they need to be expressed within the same cell. *In situ* hybridization is powerful technique to demonstrate the co-expression of a specific miRNA and associated target mRNA in a whole tissue or in specific types of cells (Martin et al. 2013; Wheeler et al. 2007). This method is further improved with use of more stable locked nucleic

acid (LNA) probes that can hybridize to the specific miRNA of interest. Other methods that can be used to investigate miRNA-mRNA possible interaction include Northern blot analysis or quantitative Real-Time PCR (q RT-PCR). While northern blot requires a large amount of isolated RNA, qPCR requires a small amount of total RNA isolate (100-200ng). Methods to detect differential protein expression encoded by the targeted mRNA include Western blot analysis, ELISA and immuno-cytochemistry experiments (Zhao et al. 2007). To prove direct interaction between a putative miRNA and its target, a reporter assay comprising a reporter plasmid containing the miRNA recognition sequence is the most reliable method to measure the reducing activity or the expression of reporter protein (Ma et al. 2007). However, methods like microarray and high-throughput sequencing provide indirect relationships between miRNAs and their targets (Lee et al. 2015). Therefore, current approaches that incorporate machine learning algorithms with literature mining for validated targets are preferably used for true target selection (Ekimler & Sahin 2014). Otherwise, the use of bioinformatics tools for targets identification followed by wet lab experimental validation can provide a more reliable method to true functional target identification.

4.1.4 Known and validated targets of miR-21

miR-21 is evolutionary conserved indicating that it is functionally important. miR-21 is overexpressed in many different types of cancer and targets numerous genes involved in cell proliferation and tumor suppression such as *PTEN*, *SPRY1*, *SPRY2, BMB, TGFb, Maspin,* (tropomyosin-1) *TPM1* and *PDCD4*. These experimentally validated miR-21 targets are summarized in Table 4-2.

Gene	Role	References
PTEN	Resisting cell death	(Ou, et al. 2014)
PDCD4	Resisting cell death/ Inflammation/ Growth suppressor/ Regulate neurite outgrowth	(Jiang et al. 2017)
TPM1	Metastasis	(Zhu et al. 2007)
SPRY1	Resisting cell death/ Metastasis	(Thum et al. 2008)
SPRY2	Resisting cell death/ Metastasis	(Mei et al. 2013)
RECK	Angiogenesis/ Metastasis	(Xu et al. 2014)
BCL2	Resisting cell death	(Sims et al. 2017)
MARCKS	Metastasis	(Li et al. 2009)
HNRPK TP63	Resisting cell death	(Papagiannakopoulos et al. 2008)
IL12A	Asthma pathogenesis	(Lu et al. 2009)
JAG1	Dendritic cell differentiation	(Ottone et al. 2014)
BTG2	Growth suppressor	(Liu et al. 2009)
LRRFIP1	Avoid immune destructive	(Li et al. 2009)
BMPR2	Resisting cell death	(Du et al. 2009)
TGFBR2	Regulating stemness	(Wang et al. 2012)
CDC25A	Sustaining proliferation	(P. Wang et al. 2009)
PELI1	Resisting cell death/ Inflammation	(Marquez et al. 2010)
ANKRD46	Metastasis	(Yan et al. 2011)
CDK2AP1	Growth suppressor	(Zheng et al. 2011)
MEF2C	Hippocampal dependent learning	(Yelamanchili, et al. 2010)
MSH6	Genome instability	(Valeri et al. 2010)
PPARA	Resisting cell death	(Sarkar et al. 2010)
FASLG	Resisting cell death	(Sayed et al. 2010)
TIMP3	Resisting cell death/ Angiogenesis/ Metastasis	(Zhang et al. 2018)
ANP32A	Resisting cell death	(Schramedei et al. 2011)
SMARCA4	Sustaining proliferation	(Schramedei et al. 2011)
THRB	Resisting cell death	(Jazdzewski et al. 2011)

Table 4-2: Examples of experimentally validated miR-21 targets.

Examples of experimentally validated miR-21 targets. The table showing some of the published miR-21 targets with emphasis on its role in apoptosis. The table is adopted from (Buscaglia & Li 2011).

Besides its role in apoptosis, miR-21 may be involved in reparative processes after traumatic brain injury (TBI) (Ge et al. 2015; Redell et al. 2011). Microarray analysis showed increased expression of mRNAs associated with sustained proliferation such as PDCD4 and PTEN through the post-transcriptional activation of the AKT signalling pathway (Han et al. 2014; Redell et al. 2011b). Additionally, miR-21 regulates blood–brain barrier (BBB) function after injury because it regulates angiogenesis molecules such as VEGF, Tie-2 and Ang-1 to alleviate BBB leakage after injury and to reduce injured brain volume (Ge et al. 2014).

4.1.5 Aims

miR-21 has been associated with many cancer types and its increased expression after brain injury is linked to better recovery (Harrison et al. 2016; Ge et al. 2014; Selcuklu et al. 2009). miR-21 can regulate a number of targets that are involved in controlling cell proliferation, cell fate differentiation and survival. The previous chapter provided evidence that supported the notion that knockdown of miR-21 in the adult hippocampus leads to decreased neurogenesis and functional deficits in spatial learning and memory via regulation of apoptosis. As it is unlikely that Caspase-3 is a direct target of miR-21, it is possible that there are other unknown pathways by which miR-21 exerts its effects on adult neurogenesis. Therefore, in this chapter we sought to investigate other potential mRNA targets and signalling pathways using two approaches. The first approach was to investigate some known miR-21 targets and pathways that are based on literature review. The second approach was to identify miR-21 targets using computational target prediction algorithms. Several different target prediction tools already exist; and we chose to

perform a bioinformatic analysis of miR-21 targets using three different databases: TargetScan, miRDB and DIANA-microT-CDS. Predicted targets from these three databases were analysed and GO cellular process and pathways involved identified. Pathways involved in miR-21 functions were analysed and associated validated targets from the literature, in relation to the brain, were investigated. Finally, genes and pathways that are possibly involved in miR-21 function in adult brain neurogenesis will be addressed.

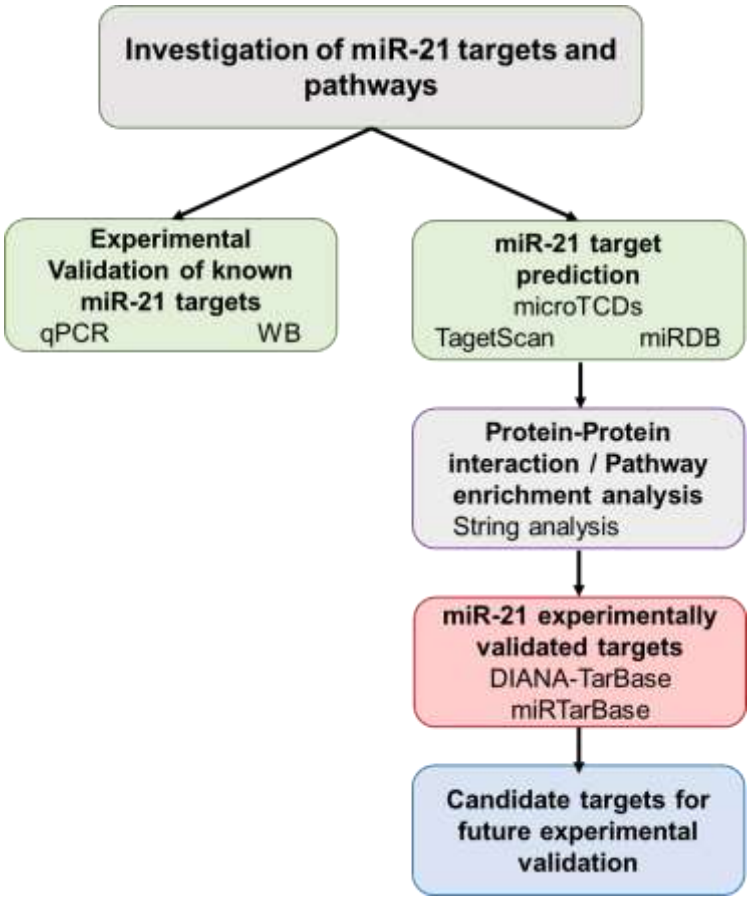


Figure 4-5: Schematic flow chart representing steps for miRNA target prediction.

A representative overview of bioinformatic tools used in the analysis, classified according to the main purpose for which they are being used. Tools that were used are presented for each step of the process.

4.2 Results

4.2.1 Altered expression of miR-21 does not affect expression of other brain-enriched miRNAs

Several miRNAs have high expression levels in the CNS; these include miR-124, miR-132 and miR-134 (Cao et al. 2016). Expression of a number of selected miRNAs (miR-128, miR-132, miR-134, miR-138 and miR-212) was examined in miR-21 OE or miR-21 KO hippocampus in order to discount the possibility that miR-21 can affect the levels of other miRNAs. MiR-128 has been reported to be involved in neural differentiation (Li et al. 2013), while miR-212 is involved in synaptogenesis (Hansen et al. 2016). Both miR-134 and miR-138 are associated with dendritic spine morphogenesis (Schratt 2009). MiR-132 has many implications for the nervous system including the regulating of neuronal differentiation, maturation, migration, axonal growth and plasticity (Qian et al. 2017). Quantitative RT-PCR analyses for the different miRNAs, using RNA extracted from hippocampal tissue, showed no significant differences in the expression of these miRNAs in relation to the overexpression or knockout of miR-21 (Figure 4-6).

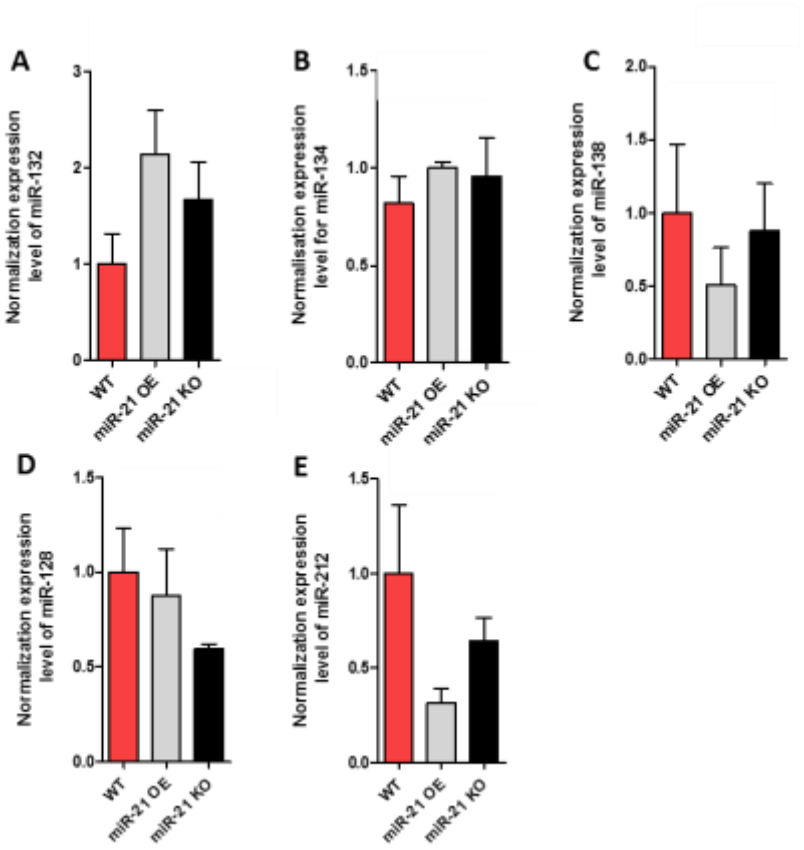


Figure 4-6: Normalized expression levels of brain-enriched miRNAs.

One way ANOVA showed no significant difference in the levels of **(A)** miR-132 ($F(2,9)= 2.146$, $p= 0.1730$), **(B)** miR-134 ($F_{(2,6)}=0.3169$, $p=0.6487$), **(C)** miR-138 ($F_{(2,6)}=0.9321$, $p=0.4441$) and **(D)** MiR-128 ($F_{(2,6)}=1.126$, p=03845) **(E)** miR-212 ($F_{(2,6)}=1.400$, $p=0.3169$) between the miR-21 overexpressing (miR-21 OE), miR-21 knockout (miR-21 KO) and wild type mice. PCR reactions were performed in triplicate and normalised to RNU6 gene. Data are plotted as +/- SEM.

4.2.1.1 Investigating downstream targets of miR-21 in the hippocampus using qPCR

We wanted to investigate whether differentially expressed miR-21 can cause significant expression changes in known miR-21 target genes. Three miR-21 target genes that have tumor suppressor inhibiting activities were selected based on extensive literature review; these included Programmed cell death (*PDCD4)*, SPROUTY2 (*SPRY1)* and transforming growth factor-β (*TGFB)* (Wachs et al. 2006; Gross et al. 2007; Gaur et al. 2011). PDCD4 is upregulated in apoptosis and downregulated in cancers (Asangani et al. 2008) . PDCD4 3'UTR has a conserved target site for miR-21 with 100% match, indicating its functional importance (Doench & Sharp 2004). *SPRY1* is a direct target for miR-21, which involve in the inhibition of the Ras/MEK/ERK pathway and regulate cell survival (Thum et al. 2008). *TGFβ* is another tumor suppressor gene that is regulated directly by miR-21 (Papagiannakopoulos et al. 2008). TGFβ signalling also increases miR-21 via TGFβR, which in turn functions in a negative feedback manner to inhibit TGF-β effects (Wang et al. 2012). TGFβR has a high binding affinity with miR-21 in numerous types of tumor including, lung, breast, colorectal, pancreatic and bladder (Rath et al. 2016).

Quantitative PCR analyses indicated that the expression levels for these three mRNAs did not change in miR-21 OE or miR-21 KO hippocampus compared to the WT hippocampus (Figure 4-7). This suggests that miR-21 did not significantly regulate the transcription of PDCD4, SPRY1 and TGFR expression. However, miRNAs act to regulate post-transcriptional levels and therefore it is possible that the lack of change in mRNA levels may not reflect a change in protein levels.

Thus, protein level measurement is necessary to identify miR-21 effect on gene expression.

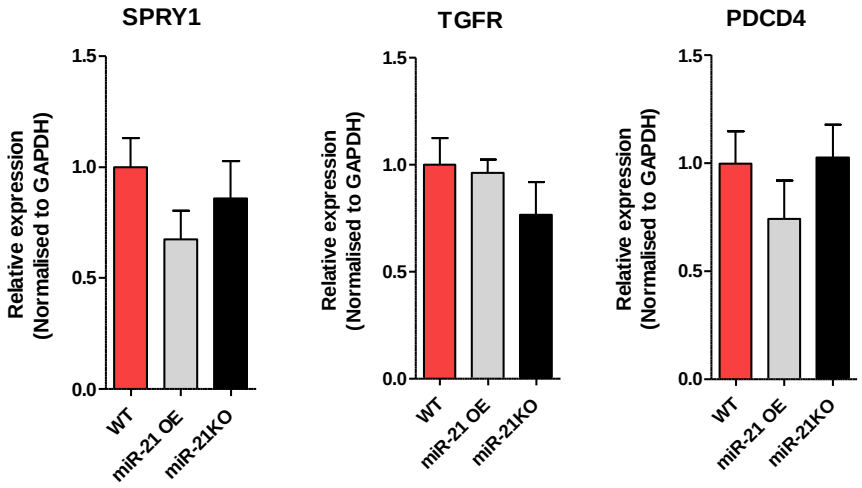


Figure 4-7: Normalised expression levels for SPRY1, TGFR and PDCD4.

One-way ANOVA showed no significant difference in the levels of SPRY1, TGFR and PDCD4. SPRY1 ($F_{(2,9)}$= 1.299, p=0.3193), TGFR ($F_{(2,9)}$= 1.117, p=0.3688) and PDCD4 ($F_{(2,9)}$= 0.9659, p=0.4169). PCR reactions were performed in triplicate and normalised to GAPDH gene. Values indicate normalised means changes in miRNA levels +/- SEM.

4.2.1.2 PTEN regulation of PI3K-AKT pathway is unaffected by miR-21

Several studies have identified the protein phosphatase and tensin homologue (PTEN) as a direct target for miR-21 in many cancer types (Wang et al. 2017) and after brain injury (Ge et al. 2015). PTEN functions by inhibiting the AKT signalling pathway as it catalyses the dephosphorylation of the 4,5-Bisphosphate (PIP2) in phosphatidylinositol 4,5-Trisphosphate (PIP3), resulting in the biphosphate product phosphatidylinositol 2 (PIP_2), thus opposing the activity of PI3K and preventing the activation of the AKT signalling pathway (Kurose et al. 2001; Panigrahi et al. 2004). AKT is one of the major cell network modulators that is involved in protein biosynthesis, cell cycle arrest and apoptosis (Azim et al. 2010).

PTEN expression levels did not change at the transcriptional level ($F_{(2,9)}$= 0.3654, p=0.7038) in miR-21 OE or miR-21 KO hippocampus compared to WT mice (Figure 4-8A). Furthermore, there were also no differences in protein levels of PTEN and phosphorylated PTEN in the miR-21 mice, compared to wild-type mice ($F_{(2,9)}$= 0.5236, p=0.6172; $F_{(2,9)}$= 0.8762, p=0.4636 respectively, Figure 4-8B-C). The levels of AKT protein was also investigated as this can be activated by the PTEN tumour suppressor. The protein levels of AKT were unchanged for miR-21 transgenic mice compared to WT (Figure 4-8D). This suggested that altered miR-21 expression in the normal brain did not mediate changes in PTEN mediated inhibition of the PI3K-AKT pathway in the mouse hippocampus.

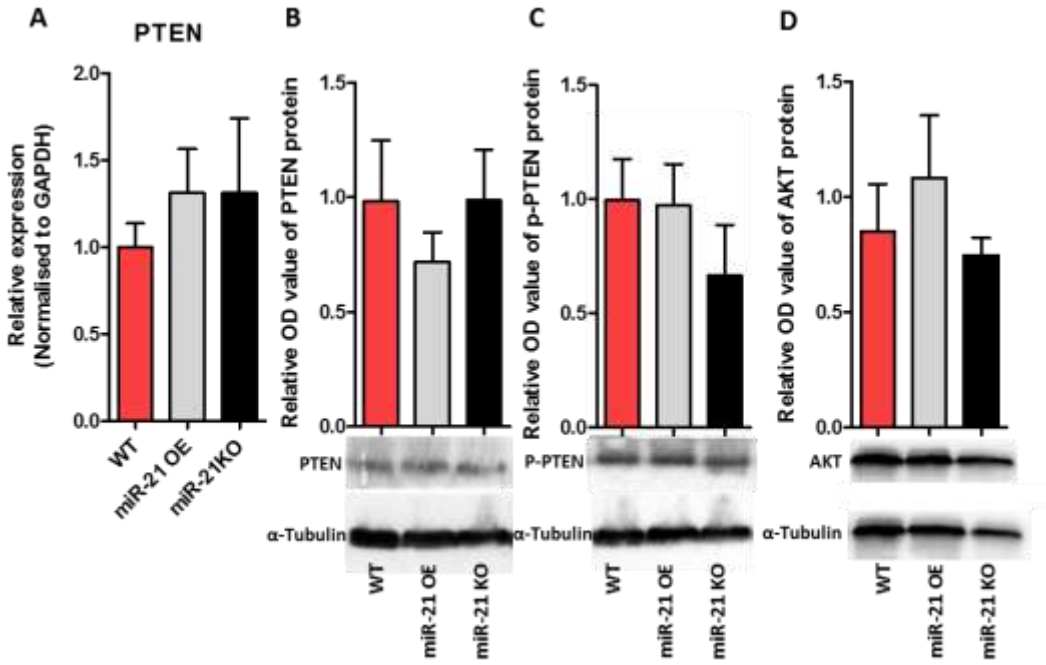


Figure 4-8: Detection of PTEN mRNA and protein levels in mouse hippocampus.

(A) The expression of PTEN was not significantly changed between miR-21 OE, miR-21 KO and wild-type mice at the post-transcriptional level when analysed by RT-PCR (n=4 per group). **(B-D)** Densiometric analysis of Western blots shown for PTEN, phosphorylated PTEN (p-PTEN) and AKT and the immunoblots for PTEN, p-PTEN and AKT with the housekeeping protein α-tubulin in hippocampal lysates (n=3 per group). One-way ANOVA was performed to determine statistical significance and values were plotted as means +/- SEM.

4.2.1.3 Wnt/ β -catenin

Wnt/β-catenin plays important roles in carcinogenesis (Zhan et al. 2017). The Wnt/β-catenin pathway is activated following signal integration from other pathways such as FGF, TGF-β, and BMP pathways, which have been found to be regulated by miR-21 in different tissues (Guo & Wang 2009). Despite no changes in TGF-β expression in miR-21 OE or miR-21 KO mice, we wanted to investigate whether miR-21 function is regulated through the Wnt signalling pathway. Western blot analysis revealed that there was no change in the β-catenin protein expression levels in miR-21 OE and miR-21 KO mice, compared to WT mice ($F_{(2, 6)}$=0.4348, p=0.6663) (Figure 4-9).

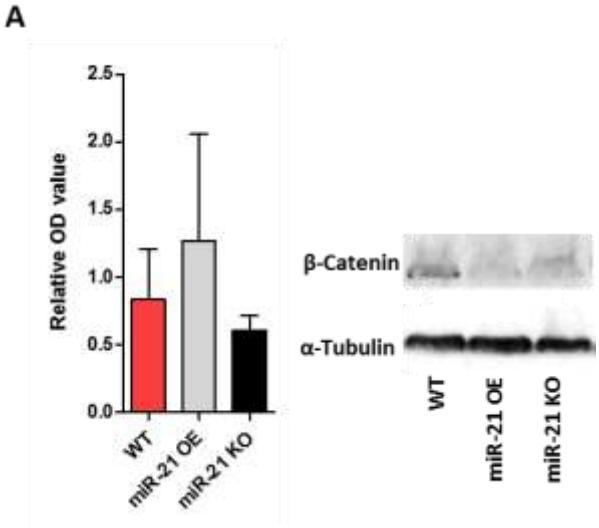


Figure 4-9: Detection of β-catenin protein levels in mouse hippocampus.

(A) Densiometric analysis of Western blots shown for β-catenin and the immunoblots for the same protein. The optical density value was normalised to the house keeping gene α-tubulin in hippocampal lysates (n=3 per group). One-way ANOVA was performed to determine statistical significance and values were plotted as means +/- SEM.

4.2.1.4 ERK and SPRY2

Extracellular Signal-regulated Kinase-1 (ERK1) and ERK2 are among the first identified networks to be regulated in cellular proliferation, differentiation and survival. MiR-21 has been reported to modulate the activity of the ERK signalling pathway through SPRY2, which is a putative target for miR-21 (Mei et al. 2013). There were no significant differences in the protein expression levels for ERK in the transgenic miR-21 mice compared to WT mice (Figure 4-10). Additionally, phosphorylated ERK (p-ERK) protein levels were investigated and again, no change was detected between groups, indicating that miR-21 functions are not regulated through the ERK signalling pathway ($F_{(2, 6)}$= 1.724, p= 0.2561, Figure 4-10A). SPRY2 is involved in the negative regulation of Ras signalling leading to glioma invasion and tumor suppressor (Kwak et al. 2011; Cabrita & Christofori 2008). Additionally, it has been reported to be targeted by miR-21 in cancer and is involved in miR-21-mediated axon growth after nerve injury (Strickland et al. 2011; Buscaglia & Li 2011a). Quantitative PCR results showed no differences in SPRY2 mRNA levels between groups ($F_{(2, 6)}$= 1.122, p=0.3673, Figure 4-10C). This was reflected in protein expression, as the levels of SPRY2 protein were not significantly different between transgenic miR-21 and WT mice ($F_{(2, 6)}$= 0.1214, p=0.8878, Figure4-10D).

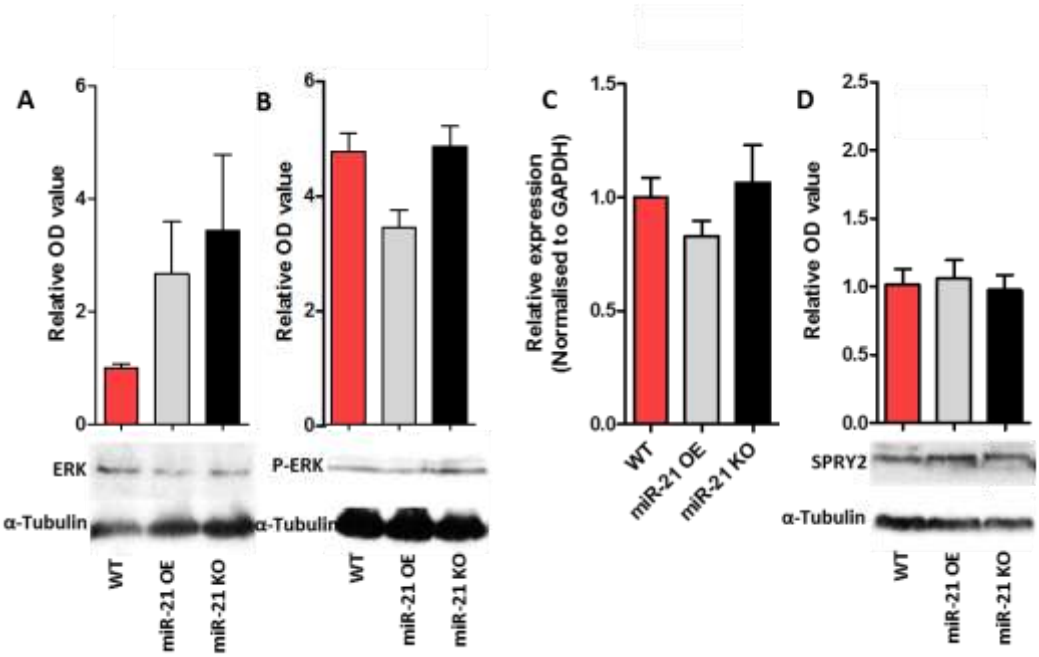


Figure 4-10: Detection of mRNA and protein expression levels for ERK, p-ERK and SPRY2 in mouse hippocampus.

(A) Densitometric analysis of Western blots shown for ERK and the immunoblots for the same protein. **(B)** Densiometric analysis of Western blots for p-ERK and the immunoblots for the same protein. **(C)** No change in the expression of SPRY2 in miR-21 KO at the post-transcriptional level when analysed by RT-PCR (n=4 per group). **(D)** Densiometric analysis of Western blots for SPRY2 and the immunoblots for the same protein. The optical density value was normalised to the house keeping gene α-tubulin in hippocampal lysates (n=3 per group). One-way ANOVA was performed to determine statistical significance and values were plotted as means +/- SEM.

4.2.1.5 Regulation of apoptotic markers by miR-21

In Chapter 3, I showed that miR-21 affects cell survival and not cell proliferation, as evidenced by increased immature and mature neurons (DCX+, Calretinin and NeuN+ cell numbers) without any changes in cell proliferation (indicated by BrdU+ and Ki67+ cell numbers) (Figure 3-3A-B). An anti-apoptotic role for miR-21 has been reported in several studies (Buscaglia & Li 2011; Gaur et al. 2011; Hu et al. 2013). Apoptosis activation leads to increased expression of the apoptotic marker Caspase-3. Therefore, it is necessary to investigate whether the miR-21 KO mice would have increased Caspase-3 protein expression. Immunohistochemistry for Caspase-3 in miR-21 OE, miR-21 KO hippocampus showed increased Caspase-3 protein levels in the DG cells of miR-21 KO mice (Figure 3-8A, B). These findings were corroborated by immunoblot analysis of hippocampal tissue, however in the latter case the increase in caspase-3 failed to reach significance ($F_{(2, 6)}$= 4.691, p=0.0594, Figure 4-11A). Nevertheless, the results indicate that Caspase-3 is highly expressed in miR-21 KO mice compared to WT mice.

Bcl2 is a pro-survival marker and has been identified as a direct target for miR-21 (Hatley et al. 2010; Yang et al. 2017; Xu et al. 2014). Bcl2 is a downstream regulator of caspase-3 activation, which is a member of the Bcl family proteins including Bax, Bad, Bid, Bcl-x_S for death inducing activity, and Bcl2 and Bcl-x_L for pro-survival activities. Increasing the intracellular pro-survival Bcl2 by preventing the release of the apoptogenic protein cytochrome c, thus preventing the activation of Caspase-3 cascade (Swanton et al. 1999). Western blotting

indicated that miR-21 OE mice demonstrated increased Bcl2 expression compared to miR-21 KO mice ($F_{(2, 6)}$= 5.277, p=0.0400, Figure 4-13B), although results failed to reach significance between miR-21 KO mice compared to WT mice. These results may indicate a possible regulatory mechanism through Bcl2 pro-survival protein associated with increased Caspase-3 expression in the miR-21 KO group.

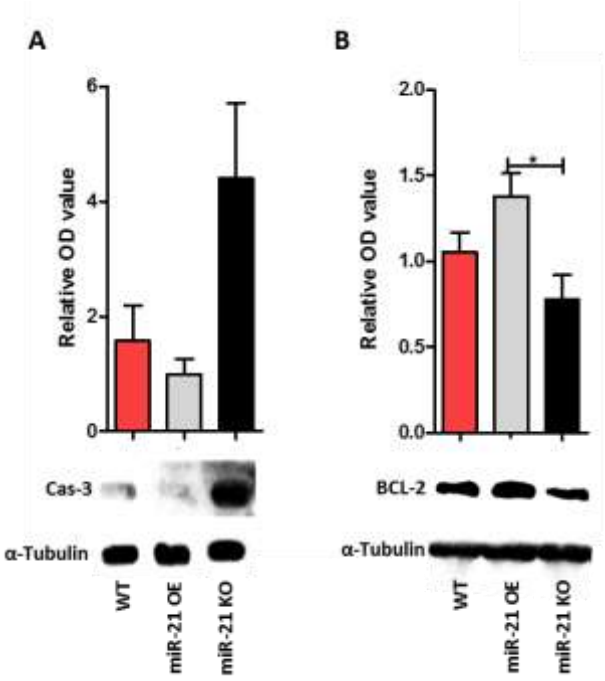


Figure 4-11: Detection of Caspase-3, BCL2 and STAT3 protein expression levels in mouse hippocampus.

(A) Densitometric analysis of Western blots shown for **(A)** Caspase-3 **(B)** Bcl 2. The optical density value was normalised to the housekeeping gene α-tubulin in hippocampal lysates (n=3 per group). One-way ANOVA was performed to determine statistical significance and values were plotted as means +/- SEM.

4.2.2 Screening of miR-21 targets using computational tools

Experimental analysis of target mRNA and protein levels in miR-21 transgenic hippocampal tissue failed to attribute a single signalling pathway to miR-21-mediated neurogenesis, although there were observations that Caspase-3 and Bcl2 protein levels were altered. To investigate if other possible target pathways involved in the regulation of miR-21 function. Computational programmes such as TargetScan, microT-CDS and miRDB were used.

4.2.2.1 miR-21 target prediction using TargetScan

A total of 302 predicted targets for miR-21-5p including conserved and poorly conserved genes were detected by TargetScan. Along with predicted targets, gene symbol, gene description, and target cumulative and total context score were reported with details and references of each target available in the table (Figure 4-12). Predicted targets obtained from TargetScan were loaded into the String analysis tool to find the protein-protein interaction and pathways involved.

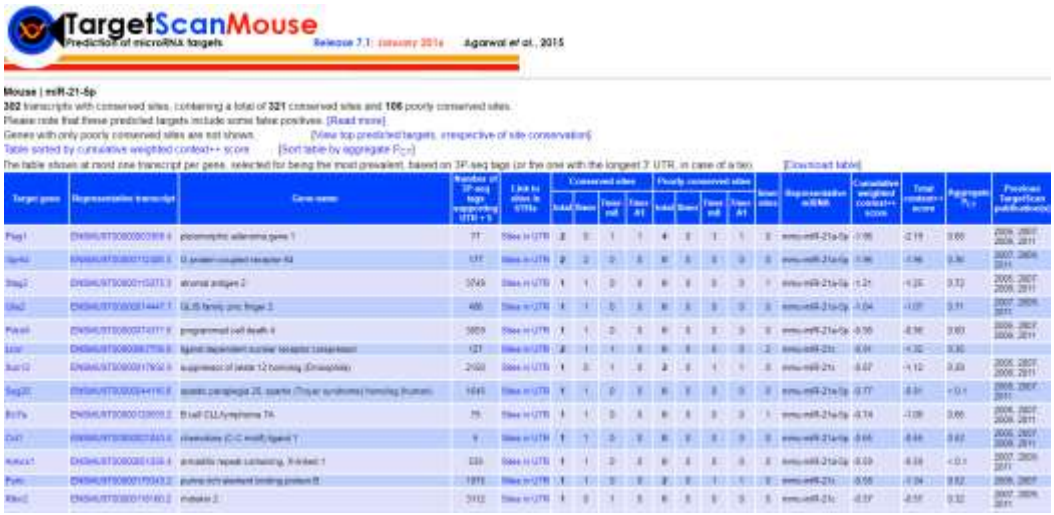


Figure 4-12: Snapshot taken from TargetScan web server

The snapshot showing some of the predicted targets for miR-21 including conserved and poorly conserved genes. Along with predicted targets, gene symbol, gene description, and target cumulative and total context score as well as publications' dates are reported.

4.2.2.2 microT-CDS v 5.0

A total of 309 targets for mir-21a-5p were selected by microT-CDS software. Targets were presented in a table; each target was given a score by the software based on factors applied by microT-CDS including seed complementarity, site accessibility and conservation (Figure 4-13). Additionally, a green square was presented if the predicted target was experimentally validated.

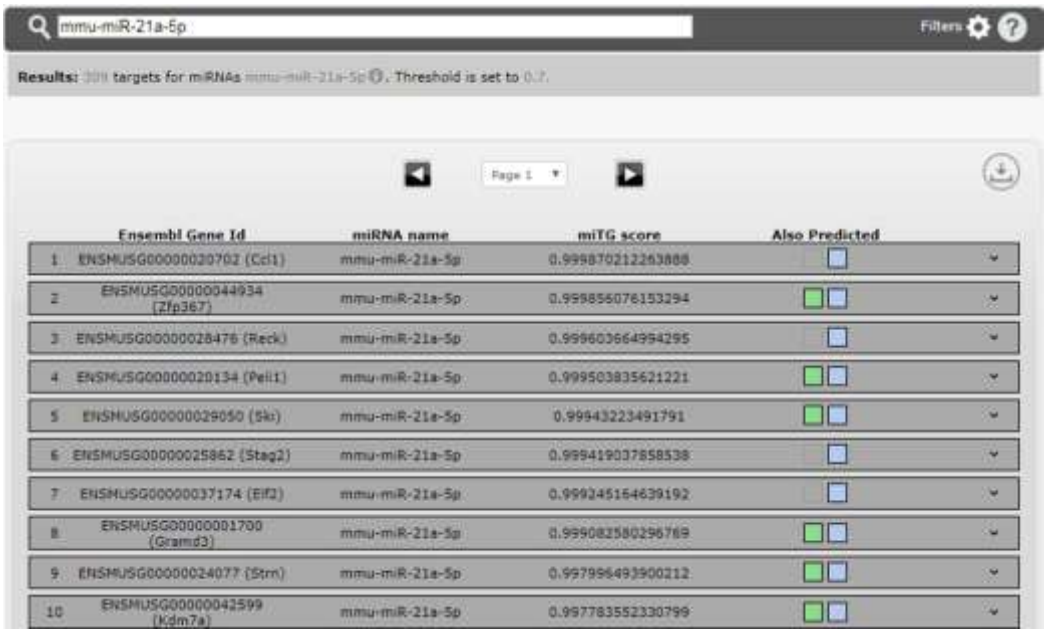
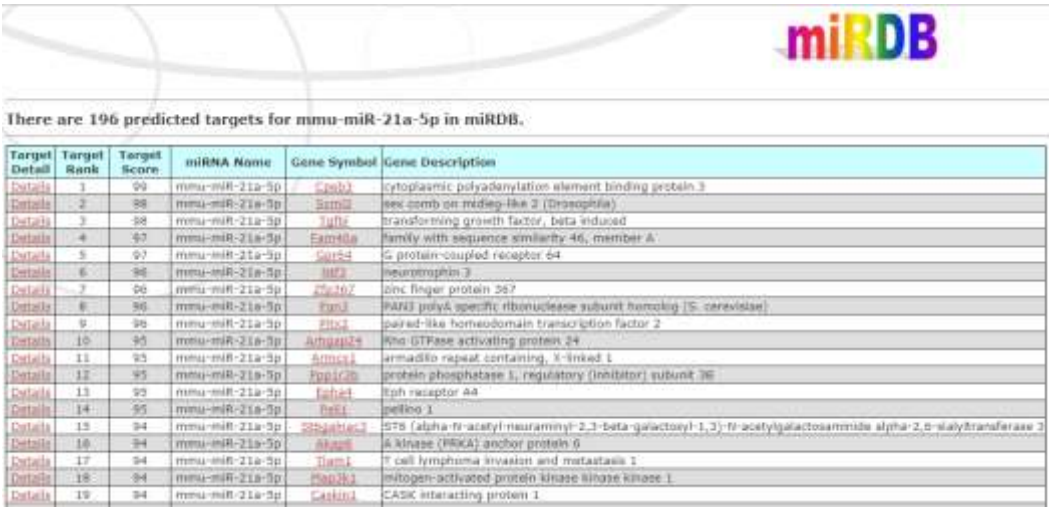


Figure 4-13: Snapshot taken from microT-CDS web server

A snapshot showing some of the predicted targets for miR-21. Along with predicted targets, gene symbol, gene description and target cumulative score are reported.

4.2.2.3 miRDB

A total of 196 predicted targets for miR-21a-5p were selected by miRDB. The targets were ordered based on their rank and score, which were given to each predicated target based on the database factors (Figure 4-14). More details are included such as the target sequence and the seed location.



There are 196 predicted targets for mmu-miR-21a-5p in miRDB.

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	99	mmu-miR-21a-5p	Cpeb3	cytoplasmic polyadenylation element binding protein 3
Details	2	98	mmu-miR-21a-5p	Scmh1	sex comb on midleg-like 2 (Drosophila)
Details	3	98	mmu-miR-21a-5p	Tgfbi	transforming growth factor, beta induced
Details	4	97	mmu-miR-21a-5p	Fam46a	family with sequence similarity 46, member A
Details	5	97	mmu-miR-21a-5p	Gpr64	G protein-coupled receptor 64
Details	6	96	mmu-miR-21a-5p	Ntf3	neurotrophin 3
Details	7	96	mmu-miR-21a-5p	Zfp707	zinc finger protein 367
Details	8	96	mmu-miR-21a-5p	Pan3	PAN3 polyA specific ribonuclease subunit homolog (S. cerevisiae)
Details	9	96	mmu-miR-21a-5p	Pitx2	paired-like homeodomain transcription factor 2
Details	10	95	mmu-miR-21a-5p	Arhgap24	Rho GTPase activating protein 24
Details	11	95	mmu-miR-21a-5p	Armcx1	armadillo repeat containing, X-linked 1
Details	12	95	mmu-miR-21a-5p	Ppp1r3b	protein phosphatase 1, regulatory (inhibitor) subunit 3B
Details	13	95	mmu-miR-21a-5p	Epha4	Eph receptor A4
Details	14	95	mmu-miR-21a-5p	Pelo	pelino 1
Details	15	94	mmu-miR-21a-5p	St8sia6os3	ST8 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3
Details	16	94	mmu-miR-21a-5p	Akap6	A kinase (PRKA) anchor protein 6
Details	17	94	mmu-miR-21a-5p	Tiam1	T cell lymphoma invasion and metastasis 1
Details	18	94	mmu-miR-21a-5p	Map3k1	mitogen-activated protein kinase kinase kinase 1
Details	19	94	mmu-miR-21a-5p	Caskin1	CASK interacting protein 1

Figure 4-14: Snapshot taken from miRDB web server

A snapshot showing some of the predicted targets for miR-21. Along with predicted targets, gene symbol, gene description and target cumulative score and rank are reported.

4.2.3 Bioinformatics analysis

Using the bioinformatics databases miRDB, DIANA TarBase and TargetScan, computationally predicted targets for miR-21a-5p were generated. A list of predicted targets from each database was then loaded separately into STRING to identify possible protein-protein interactions. GO analysis for gene ontology biological processes and KEGG analysis for associated pathways were also obtained (Figure 4-15A-B). Top three GO biological processes were regulation in metabolic processes, regulation of macromolecules metabolic processes and regulation of gene expression. The top KEGG pathway associated with the predicted targets generated by miRDB, Tarbase and TargetScan was the MAPK signalling pathway as it was a common pathway in all three bioinformatics databases.

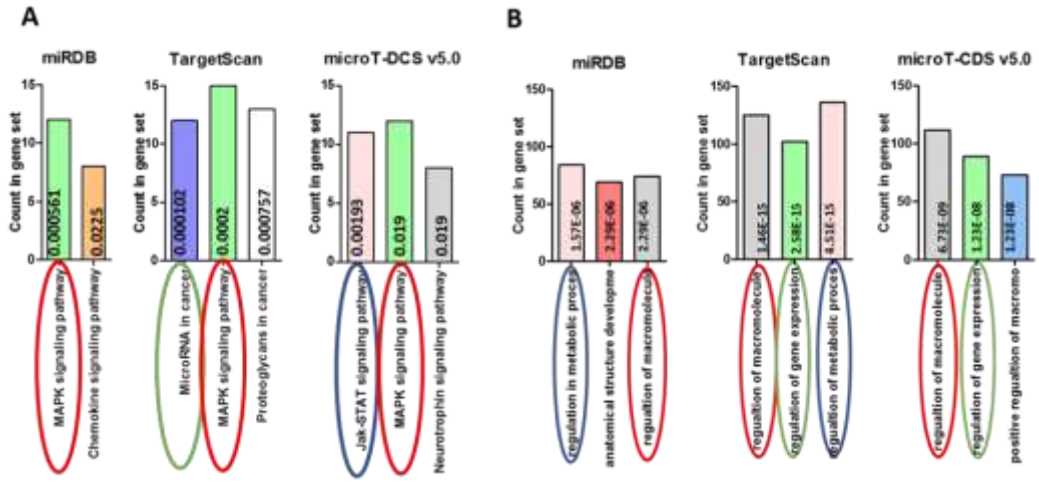
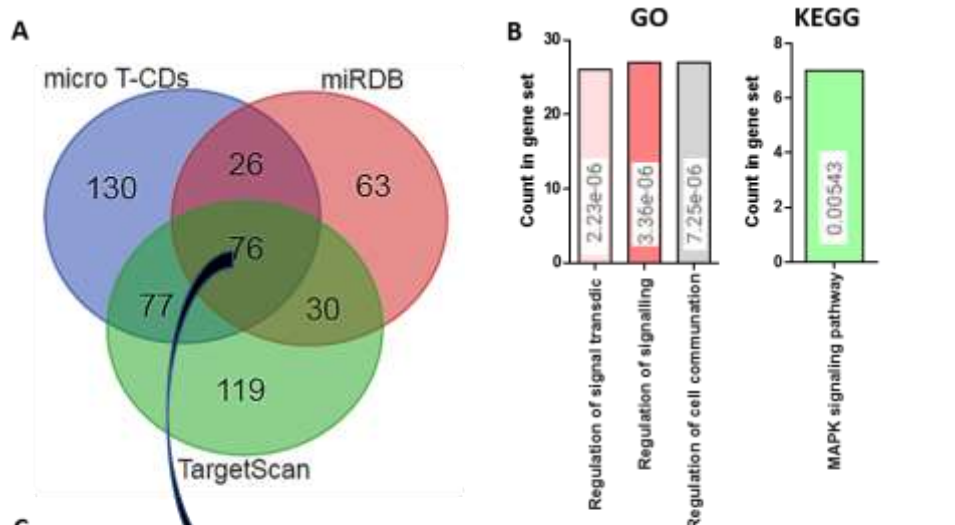


Figure 4-15: Bioinformatics analysis using miRDB, TargetScan and microT-CDS by Diana tools.

(A) The top three GO biological processes associated with the miR-21a-5p targets. **(B)** The top three KEGG pathways associated with the miR-21a-5p targets predicted.

4.2.3.1 Comparison btween miRDB, microT-CDS v 5.0 and TargetScan

Targets predicted by each program were inserted into Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/) to find common predicted targets between the three databases to increase the probability of finding 'true' miR-21 targets. Targets predicted by these three databases were quite different, with 76 targets matched in all three databases (Figure 4-16A-B). However, despite the selected databases were updated recently, few well known validated targets were missing from the group of common targets such as PDCD4 and PTEN (Figure 4-16C). A list of these common target and their function are in the appendix. Therefore, the prediction algorithm for computationally predicted targets databases needs to be further addressed.



Names	total	elements
TargetScan miRDB micro T-CDs	76	Gid4 Jph1 Zfp367 Suz12 Gpr64 Mprip Armcx5 Srl Crebrf Pcbp2 Plekha1 Caskin1 Rasgrp1 Map3k1 Tism1 Jag1 Ubr3 Nfib Scml2 Rspo2 Fasl Map2k3 Klf6 Hipk3 Glcci1 Abi1 Ntf3 Pja2 Yap1 Rab11a Armcx1 Stag2 Gramd3 Tgfbi Arhgap24 Fam46a Ccl22 Fubp1 Spry1 Acat1 Krit1 Peli1 Kdm7a Arhgef12 Ccl1 Pag1 Dkk2 Nfia Zfp704 Edrf1 Mef2c Ski Dusp8 Spry2 Slmap Tmem170 Pbrm1 Il21 Adtrp Ehd1 Wwp1 Epha4 Ube2d3 Ppp1r3a Pfkm Elf2 Reck Pik3r1 Smad7 Chic1 Yod1 Thrb Klhl42 Pcsk6 Pcbp1 Slc30a10

Figure 4-16: Bioinformatics analysis for miR-21 target predicted genes.

(A) Venn diagram representation performed on target predicted by miRDB, micro T-CDs and TargetScan. **(B)** The subpopulation of genes commonly regulated by miR-21-5p expression in the three databases, Gene ontology. Number on each column represents the false discovery rate (GO), and KEGG analysis was performed using STRING database. **(C)** Table showing the common 76 targets in the Venn diagram illustrated in (A).

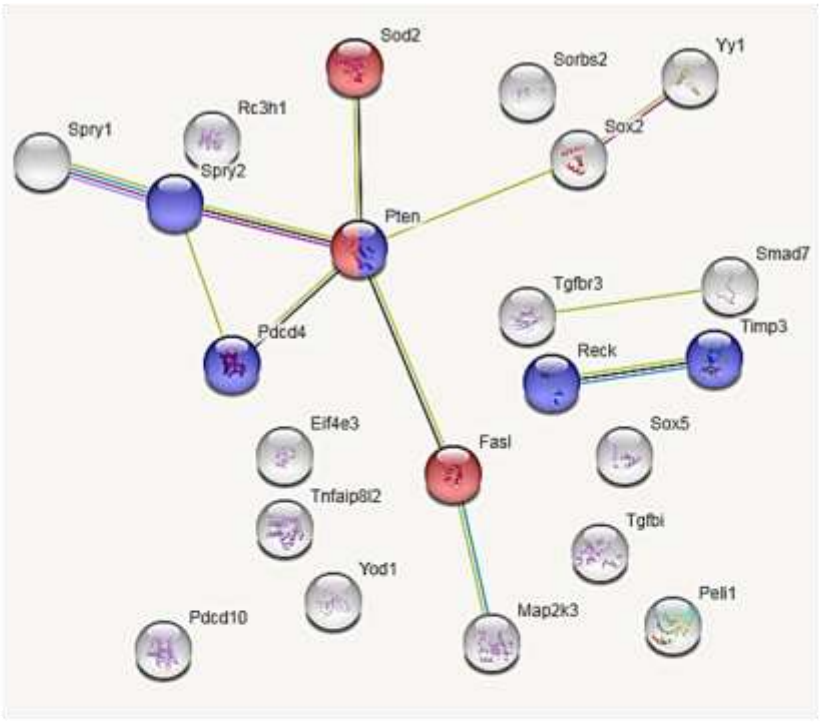
4.2.4 Experimentally validated targets

To investigate the validated target feature using specific programmes for experimentally validated targets, miRTarBase and DIANA TarBase v 8., which is part of the DIANA-Tool webpage, to find out if the identified targets are more specific and sensitive in comparison to miR-21 targets identified by TargetScan, miRDB and microT-CDs v.0.5 (programmes with no experiment validation feature).

4.2.4.1 miRTarBase http://miRTarBase.mbc.nctu.edu.tw/

Using miRTarBase (http://miRTarBase.mbc.nctu.edu.tw/), 41 validated targets were obtained from the *Mus musculus* catalogue. Validation methods were grouped as strong and less strong: target site reporter assays, target measurements such as Western blot or qPCR, were strong evidence while microarrays, NGS, pSILAC, and others were less strong evidence. In total, 28 genes out of the 41 genes that were identified were supported by strong evidence, and 13 genes were identified by less strong evidence. Several target genes were reported by more than two publications including *SPRY1, SPRY2, SPRY3, SPRY4, PDCD4, PELI, RECK, PTEN* and *SMAD7*. However, publication provided in the table cannot be checked manually. Targets supported by strong evidence were inserted into STRING analysis tool to obtain protein-protein interactions and pathways involved. The top significant pathways involved in the target list were microRNAs in cancer and FoxO signalling pathways (Figure 4-17).

A



B

Biological Process (GO)			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0008285	negative regulation of cell proliferation	9	1.79e-06
GO:0009966	regulation of signal transduction	14	1.79e-06
GO:0048583	regulation of response to stimulus	15	1.79e-06
GO:0060255	regulation of macromolecule metabolic process	18	1.79e-06
GO:0065009	regulation of molecular function	14	1.79e-06
			(more ...)

KEGG Pathways			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
05206	MicroRNAs in cancer	5	5.55e-05
04068	FoxO signaling pathway	3	0.0398

Figure 4-17: Validated targets analysis using miRTarBase.

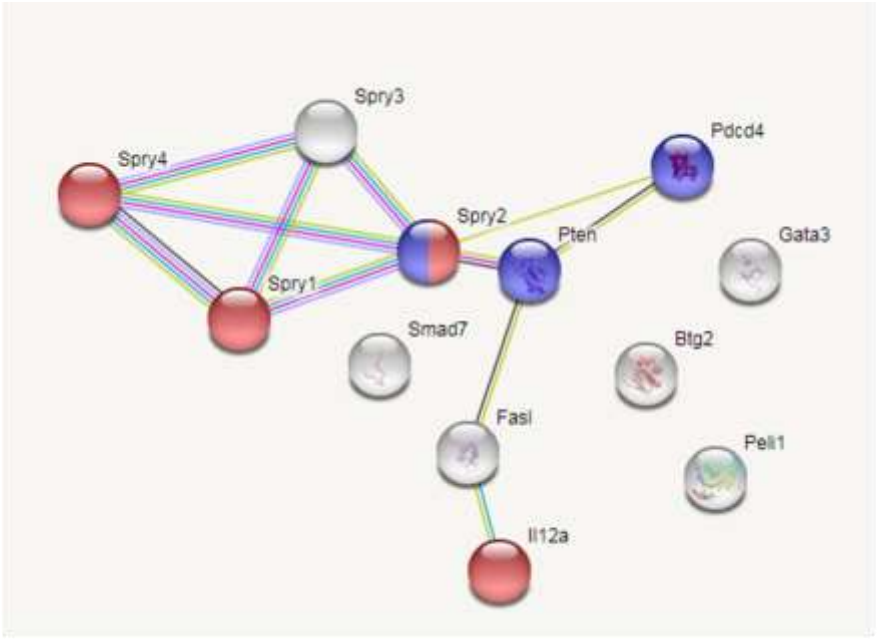
(A) Schematic representation of the 41 experimentally validated genes implicated in molecular interactions, using the STRING software. **(B-C)** The circles are coloured based on the colour of the GO biological process and the KEGG signalling pathway. The width of the lines represents molecular interaction between genes.

4.2.4.2 DIANA TarBase v. 8 (http://www.microrna.gr/tarbase)

The TarBase web programme consists of species, experimental methodology and a prediction score for each gene. Additionally, experimental conditions and validation type including direct and indirect interaction were provided in the filtering column. DIANA TarBase v.8 was updated in January 2018, and it has the most comprehensive database with about 670000 miRNAs:target interactions (Karagkouni et al. 2018). Using DIANA TarBase v.8, 2224 interactions were obtained with miR-21 from 24 experiments, 9 using low input methods such as qPCR, Western blot and luciferase reporter assay (strong evidence) and 15 experiments using high input methods such as microarrays, RNA seq and mass spectrometry. These targets were obtained from 10 publications published between 2008-2014, despite the web programme was last updated in January 2018, indicating the need of including more recent studies.

Fourteen genes out of the 2224 interactions were supported with low input. These genes were inserted into STRING analysis to obtain protein-protein interaction and pathways involved. The top pathways involved were Jak-STAT and microRNA in cancer (Figure 4-18).

A



B

Biological Process (GO)			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0032268	regulation of cellular protein metabolic process	10	6.46e-06
GO:0051348	negative regulation of transferase activity	6	6.46e-06
GO:0071901	negative regulation of protein serine/threonine kinase activity	5	6.46e-06
GO:0008285	negative regulation of cell proliferation	7	6.7e-06
GO:1902531	regulation of intracellular signal transduction	8	3.23e-05
			(more ...)

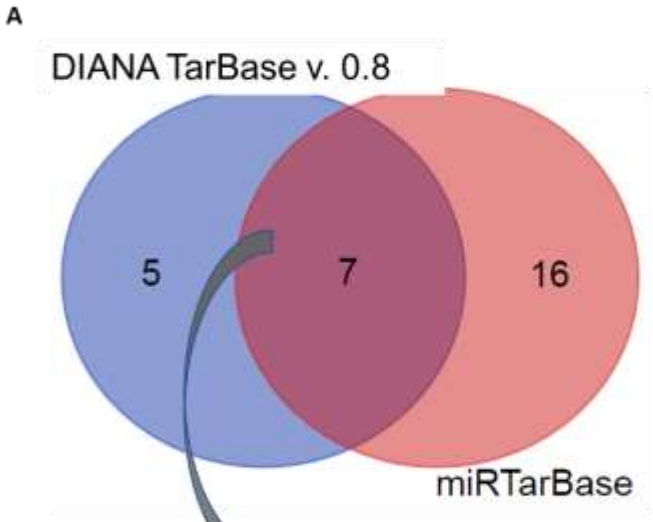
KEGG Pathways			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
04630	Jak-STAT signaling pathway	4	0.000248
05206	MicroRNAs in cancer	3	0.00677

Figure 4-18: Validated targets analysis using TarBase.

(A) Schematic representation of the 41 experimentally validated genes implicated in molecular interactions, using the STRING software. **(B-C)** The circles are coloured based on the colour of the GO biological process and the KEGG signalling pathway. The width of the lines represents molecular interaction between genes.

4.2.4.3 Comparison between low input methods validated targets obtained from miRTarBase and DIANA TarBase V. 8

Targets predicted by both programs: miRTarBase and DIANA TarBase V. 8 were inserted into Bioinformatics & Evolutionary Genomics to find common predicted targets. Seven targets were common between both programs including, *Spry1*, *Spry2*, *Pten*, *Pdcd4*, *Fasl*, *Peli* and *Smad7* (Figure 4-19). All these targets have been published in miR-21 related studies (Buscaglia & Li 2011; Luo et al. 2017).



B

Names	total	elements
DIANA Tool TarBase v.08 miRTarBase	7	Spry1 Spry2 Pten Pdcd4 Fasl Peli1 Smad7
DIANA Tool TarBase v.08	5	Il12a Btg2 Spry4 Spry3 Gata3
miRTarBase	16	Sox5 Tgfbi Yy1 Tnfaip8l2 Gt(ROSA)26Sor Reck Eif4e3 Tgfbr3 SORBS2 Sod2 Rc3h1 Timp3 Sox2 Map2k3 YOD1 Pdcd10

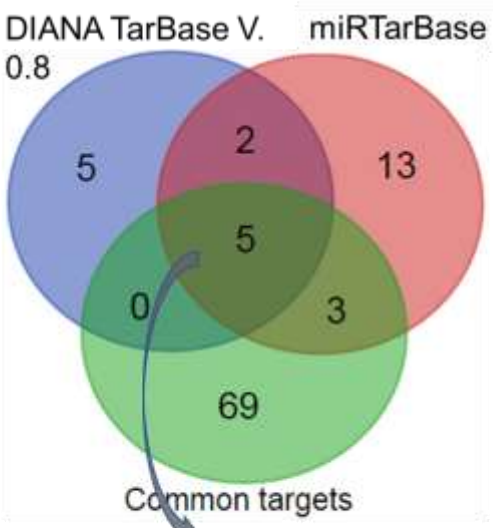
Figure 4-19: Comparison between validated target obtained from miRTarBase and TarBase V.8.

(A) Venn diagram representation performed on validated targets obtained from miRTarBase and TarBase V.8, and published target **(B)** The subpopulation of common genes in each subgroup of the Venn diagram.

4.2.4.4 Comparison between low input methods validated targets obtained from miRTarBase and DIANA TarBase V. 8 and common targets by miRDB, micro T-CDs and TargetScan

Targets predicted by low input methods validated targets (Section 4-2-4-3) and common predicted targets obtained from the three programs: miRDB, micro T-CDs and TargetScan (Section 4-2-3-1) were inserted into Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/) to find common predicted targets between the three databases and experimentally validated targets. Out of the 7 common targets between both validated targets programs, 5 targets were common with 76 list of common targets identified by the three prediction programmes including, Spry2, Fasl, Smad7, Spry1 and Peli (Figure 4-20). Despite targets obtained by miRDB, micro T-CDs and TargetScan programs and validated targets, had some similarities, nevertheless, many did not match. This may result because genes selected by the three prediction programs, were identified by low and high input methods.

A



B

Names	total	elements
Common targets DIANA Tool TarBase v.08 miRTarBase	5	Spry2 Fasl Smad7 Spry1 Peli1
DIANA Tool TarBase v.08 miRTarBase	2	Pten Pdcd4
Common targets miRTarBase	3	Map2k3 Tgfbi Reck
DIANA Tool TarBase v.08	5	Btg2 Spry4 Il12a Spry3 Gata3
miRTarBase	13	Sod2 Rc3h1 Sox5 Tnfaip8l2 Eif4e3 Timp3 YOD1 Pdcd10 Yy1 Gt(ROSA)26Sor Tgfbr3 Sox2 SORBS2
Common targets	69	Pik3r1 Crebrf Pcbp2 Ski Yap1 Dusp6 Adtrp Glcci Ehd1 Kdm7a Rab11a Plekha1 Gid4 Caskin1 Arhgef12 Chic1 Stmap Ccl1 Jph1 Pag1 Cbep3 Armcx1 Wwp1 Rasgrp1 Epha4 Stag2 Klf8 Ube2d3 Ppp1r3a Dkk2 Gramd3 Hipk3 Yod1 Pfkm Nfia Map3k1 Thrb Arhgap24 Zfp704 Zfp367 Tiam1 Suz12 Elf2 Gpr64 Klhl42 Mprip Jag1 Fam46a Ccl22 Tmem170 Fubp1 Ubr3 Pcsk6 Nifb Pcbp1 Armcx5 Slc30a10 Alx1 Pbrm1 Acat1 Scml2 Krit1 Rapo2 Ntf3 Pja2 Srl Edrf1 Il21 Mef2c

Figure 4-20: Comparison between validated targets and common predicted targets.

(A) Venn diagram representation performed on validated targets obtained from miRDB, micro T-CDs and TargetScan. **(B)** The subpopulation of common genes in each subgroup of the Venn diagram.

4.3 Discussion

In this chapter, possible targets for miR-21 using experimental approaches such as immunohistochemistry (IHC), qPCR and WB, as well as using computational target databases were explored. In order to identify possible targets and pathways of miR-21 in the normal brain, the expression of a number of reported targets for miR-21 in cancer, TBI and SCI such as *Pten*, *Spry2*, *Bcl2* and *Stat3* were investigated. Many of these targets did not show differences in expression in the transgenic miR-21 hippocampus compared to WT.

MiR-21's role in cancer and TBI has been investigated (Chan et al. 2005; Du et al. 2009; Miguel-Hidalgo et al. 2017; Redell et al. 2011; Han et al. 2014). The role of miR-21 in the regulation of cell proliferation in cancer is through inhibition of several target genes including *Spry1, Spry2, Btg2* and *Pdcd4*. Therefore, it was hypothesised that these proteins would be upregulated in miR-21 KO hippocampus. Our experimental analysis showed no significant changes in levels of mRNAs for *PTEN, SPRY1, TGFB and PDCD4* in miR-21 OE or miR-21 KO mice, compared to WT mice. However, as miRNAs can act to alter protein levels without changing mRNA levels, measurement of protein expression is needed to further validate possible targets. PTEN/AKT signalling pathway was then analysed as this pathway influences the biological behaviors of malignant cells and is associated with proliferation, expansion, metastasis and survival (Yan-nan et al. 2014; Gross et al. 2007). Analysis indicated no differences among different miR-21 groups, suggesting that miR-21 does not regulate the PTEN/AKT pathway in the brain (Figure 4-7).

The MAPK/ ERK pathway which is involved in stem cell fate determination and many other cellular processes was investigated (Hanafusa et al. 2002; Li et al. 2013; Ritt et al. 2016). SPRY2 is a direct target for miR-21, and a negative regulator of the ERK signalling pathway (Chandramouli et al. 2008). It has been reported that increased expression of SPRY2 inhibits the differentiation and survival processes through the negative feedback loop regulation of receptor tyrosine kinase signalling pathway in mature neurons (Gross et al. 2007). It was hypothesised that miR-21 regulates ERK pathway by targeting SPRY2. Additionally, the up-regulation of miR-21 could reduce the repression of ERK/MAPK signalling mediated by SPRY2 to maintain ERK/MAPK activation during NPC differentiation. Investigation of ERK and SPRY2 protein expression levels showed no significant differences in miR-21 transgenic mice.

The Wnt/ β-catenin signalling pathway is a highly conserved pathway that is involved in the regulation of nervous system development (Zechner et al. 2003; Hari et al. 2002). The pathway has many implications in cell proliferation, differentiation and apoptosis and can lead to tumorigenesis (Wu et al. 2015). Additionally, the disruption of the Wnt/ β-catenin pathway may result in the development of many CNS diseases including schizophrenia, mood disorders and autism, and neurodegenerative diseases such as AD (Lovestone et al. 2007; Inestrosa & Varela-Nallar 2014). Additionally, increased Wnt signalling *in vivo* by lentiviral injection into the adult rat hippocampus increases adult hippocampal neurogenesis (Lie et al. 2005). In this study, miR-21 overexpression or knockdown did not have any effects on β-catenin protein levels in the hippocampus.

It has been reported previously that miR-21 inhibition results in the activation of caspase-3 which results in cell apoptosis (Chan et al. 2005). Measurement of caspase-3 protein levels showed increased expression in miR-21 KO mice (Figure 4-11A). However, caspase-3 is not directly targeted by miR-21 as the caspase-3 3'UTR does not have a miR-21 binding site, and the 3' UTR luciferase reporter assay is not suppressed by miR-21 (Hatley et al. 2010). Therefore, it is likely that caspase-3 downregulation by miR-21 is an indirect consequence of unidentified target of miR-21.

In contrast, BCL2 is a direct target for miR-21 and has an important role in cell survival through the interaction with other members in the BCL2 family including cell death agonist Bax, Bad and Bid and cell death suppressors including Bcl-X_L, Mcl-1 and Bcl-w Bcl2 (Huang 2002). Detection of this pro-survival protein in the miR-21 OE and miR-21 KO groups compared to the WT group demonstrated an increase in BCL2 expression in miR-21 OE and WT tissue compared to miR-21 KO tissue, despite results failing to reach significance between miR-21 KO group and WT group. Nevertheless, this suggests that BCL2 may be involved in the regulation of miR-21 function.

Despite the fact that caspase-3 and BCL2 protein expression was dysregulated in the miR-21 KO compared to the WT hippocampus, the results failed to reach significance. Our experimental results may be attributed to using whole hippocampal tissue for qPCR and WB analysis instead of the DG, which may have diluted the expression of proteins involved. The use of DG tissue will yield a smaller quantity of mRNA and protein but may provide more sensitivity.

The failure to experimentally validate known miR-21 targets suggested the need for a different approach. To predict other possible miR-21 targets, bioinformatics tools were applied. Bearing in mind that experimental validation of possible miRNA targets can be time consuming and an expensive endeavour, computational tools may be the way forward. Using a combination of three bioinformatics prediction tools namely TargetScan, miRDB and Diana TarBase, a number of miR-21 were identified. Previously it was concluded that targets predicted by more than one program are true targets, as a combination of algorithms can result in increased sensitivity of detection (Riffo-Campos et al. 2016). It has been reported that the use of five different bioinformatics programs including TargetScan, TargetScanS, PicTar, EIMMO and DIANA-microT-CDs had increased target predicted specificity sensitivity (Gaidatzis et al. 2007; Lim et al. 2005a). Thus, TargetScan and DIANA-microT-CDs were among the selected computational prediction databases. In addition, the miRDB database was among the selected prediction tools due to the relatively recent update of the database (2015), with a large number of miRNAs (~7000), in different animal species (Wong & Wang 2015).

Using the three databases, 76 common genes were predicted to be targeted by mmu-miR-21-5p. Analysis of the detected targets indicated high similarities with published studies related to miR-21 targets. Literature mining of miR-21 targets indicated some common targets with the prediction programmes, for example, *Spry2, Fasl, Rasgrp1, Tgf, Thrb, Jag1, Reck, Spry1, Peli1* and *Mef2c*. Nevertheless, some known targets were missing from the list including *PDCD4,*

PTEN and *STAT3*, while other genes have not been investigated such as *Ehd1, Sr1* and *Dkk2*. The later potential target group can be regulated by miR-21 under normal conditions. The biological process gene ontology (GO) classification and KEGG pathway analysis of the common predicted target genes, suggested that miR-21 is involved in the regulation of numerous processes of the cellular function and that miR-21 regulates target proteins associated with MAPK signalling pathway (Figure 4-16).

To investigate the experimentally validated feature included in the bioinformatics tools, analysis of miR-21 validated targets was conducted using miRTarBase and DIANA TarBase v.8.0. In total, 2224 validated interactions were detected by DIANA TarBase v. 8, and 41 validated targets were detected by miRTarBase. Both pieces of software have been updated recently and included more targets which have been collected from published literature, and more added features related to the method of validation, species and type of tissue used etc. It has been reported that DIANA TarBase is the largest available and most comprehensive validated prediction database (Lee et al. 2015); nevertheless, miRTarBase has more than 360000 miRNAs-Target interactions including coding and non-coding RNAs (Moore et al. 2015; Chou et al. 2018), which makes the use of both databases a more comprehensive approach. Analysis of miR-21 validated targets using low input methods in both tools showed some differences. Validated interactions by DIANA TarBase detected 12 miR-21 target interactions, while miRTarBase detected 23 targets. Comparison of the two revealed 7 targets that were common: *Spry1, Spry2, Pten, Pdcd4, Fasl, Peli* and *Smad7* (Figure 4-18).

Literature mining using review studies with miR-21 targets and using biomedical literature mining serves (http://finder.sciride.org/), identified that both programs need further updating, as some validated targets were missing from both tools (Krichevsky & Gabriely 2009; Buscaglia & Li 2011a; Volanakis & Krawczyk 2018).

At present the mechanism by which miR-21 regulates neurogenesis is still unclear. Potential miR-21 pro-survival regulatory function have been identified. The investigation of the expression levels of other apoptotic genes, including *Pdcd4*, and *Fasl*, which were among common validated targets selected by DIANA TarBase V. 8 and miRTarBase, may explain increased cell apoptosis when miR-21 is deleted (Liu et al. 2011; Gaur et al. 2011; Kangas et al. 2017). Additionally, investigation of other apoptotic proteins that are targeted by miR-21, for example Fasl, can further explain which apoptotic pathway miR-21 regulates. The trigger of external apoptotic pathway by Fasl results in the activation of death receptors cascade such as tumor necrosis factor (TNF) and Fas, leading to the activation of capspase-8, which results in the activation and cleavage of caspase-3, thus resulting in apoptosis. On the other hand, the intrinsic pathway is regulated by different types of cell stress factors such as BAX and BAK proteins, resulting in the release of cytochrome c and activation of Apaf1 protein, which mediates the expression of caspase-9, thereby activating cleaved caspase-3 and leading to apoptosis. The two mechanisms are regulated through the activity of pro-survival protein Bcl2 and $Bclx_L$ (Mohammad et al. 2015). Therefore, the reduction in Bcl2 protein expression in miR-21 KO mice can explain our caspase-3 results, as

caspase-3 is a downstream factor for Bcl2, and both proteins participate in the both apoptotic pathways.

Comparison of the bioinformatics results with reported literature led to identification of a number of proteins and signalling pathways. Proteins involved in the regulation of NPC survival and differentiation were highlighted including, TGF-β, Jag1 and Fasl (Ming & Song 2011). Additionally, other proteins were selected such as Myocyte enhancer factor 2C (Mef2c), which has a role hippocampal-dependent learning and memory and EH Domain Containing 1 (Ehd1), which has a role in neurite outgrowth. Further analysis of these proteins may provide more information on the mechanism of miR-21 mediated neurogenesis.

Using different databases to identify miR-21 targets can allow novel networks involving miR-21 to be studied. However, there is no universal target prediction algorithm to specifically identify miRNA targets, and solely relying on computational analysis based on different predicted genes obtained from each bioinformatics predicted programme is not reliable. Although some miRNA may have potential target binding sites, nevertheless, these may not be functional and will need to be validated experimentally (Seitz 2009; Helwak et al. 2013). By the same token, some poor seed regions that are not be predicted by algorithms are well characterised by miRNA: mRNA experimental interactions (Lal et al. 2009). This indicates that depending on the seed region as the main criteria for miRNA: target interaction makes the sole use of bioinformatics approaches doubtful.

Attempts to identify possible miR-21 regulatory targets using protein expression did not conclusively ascertain the direct target(s) of miR-21 that are linked to adult neurogenesis in the miR-21 transgenic hippocampus. The negative results obtained in the experimental part may be attributed to high noise to signal ratio in the whole hippocampal sample used. Due to time constraints, we were unable to pursue validation of other targets that could be important, for example apoptotic proteins such as, Apf1a, Fasl, Pdcd4 and Tgfβ. In addition to experimental target recognition by qPCR and WB approaches, it will also be vital to incorporate other experimental approaches such as reporter assays and blocking of selected genes *in vivo* and *in vitro* to fully establish miR-21 regulated mechanisms in the brain.

In conclusion this chapter provided potential miR-21 mechanisms for increased NPC apoptosis in miR-21 KO animals. Additionally, a number of genes and pathways have been suggested for future investigation as it would be interesting to identify pathways involved in miR-21-mediated apoptosis when its expression is knocked down. This chapter also highlights the importance of incorporating bioinformatics tools with wet lab experiments to identify 'true' miRNAs targets. Using several bioinformatics tools to identify common putative targets is potentially a sound strategy, as more selective of which targets to experimentally validate to elucidate signalling networks that are regulated by miRNAs during cellular processes. This approach can enable the identification of factor(s) involve in regulating NPC survival. In the longer term, understanding these pathways may help uncover new targets that can be developed clinically to promote neuronal survival in diseased brains.

Chapter 5 The role of miR-21 in adult subventricular zone neurogenesis

5.1 Introduction

Adult neurogenesis is a dynamic process and involves the production of new neurons from adult neural progenitor cells (NPC) in specific regions throughout life. These newly generated neurons are able to interact with the existing neural circuit in a process regulated by special properties of adult NPCs and the microenvironment niche. The subventricular zone (SVZ) In the adult brain is one of the neurogenic regions associated in adult neurogenesis, the other being the dentate gyrus (DG). Several types of cells are involved in the process of SVZ-adult neurogenesis, including ependymal cells which line the lateral ventricles, glia-like stem cells (type B), transient amplifying cells (type C) which are derived from type B cells and divide rapidly to give rise to immature neuroblasts (type A cells) that migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into distinct types of GABAergic and dopaminergic interneurons (Bonzano et al. 2016).

Adult neurogenesis has been shown to be regulated by internal and external mechanisms within the neurogenic niches in the adult brain. Non-coding RNAs, including microRNAs, have recently been shown to be involved in the regulation of the three main stages of adult neurogenesis: proliferation, differentiation and survival. This regulation comes about because of their ability to inhibit translation and/or stimulate degradation of target genes that are involved the proliferation and differentiation of many types of stem cells, thus affecting the neurological niches (Cheng et al. 2009; Jin et al. 2016; Han et al. 2016; Papagiannakopoulos & Kosik 2009; Bielefeld et al. 2017).

5.1.1 The role of miRNAs in adult SVZ neurogenesis

Current microRNA research seeks to understand their activities in the progression of different neurodegenerative diseases and to identify their utility as associated molecular markers (Sun & Shi 2014). With regards to generating newborn neurons in the SVZ, previous reports have linked several miRNAs to the regulation of adult neurogenesis. For example, miR-124, which is a highly abundant miRNA in the CNS, has been found to be associated with increased SVZ neurogenesis. Upregulation of miR-124 in type C cells and type A cells in the SVZ resulted in increased neurogenesis through its regulation of SRY-box transcription factor Sox9 (Cheng et al. 2009). In addition, miR-137, which is also a highly abundant microRNA in the CNS, is regulated by MeCP2, an epigenetic DNA methyl-CpG–binding protein, leading to modulation of proliferation and differentiation of adult NPCs in the SVZ (Szulwach et al. 2010). MiR-25 function has been investigated in murine NPCs derived from primary adult SVZ cultures. It has been found that knocking down miR-25 expression decreased NPC proliferation, whereas increased expression of miR-25 stimulated NPC proliferation, through the regulation of insulin-like growth factor 1 (IGF1) (Brett et al. 2011). Additionally, a study found that increased miR-410 expression inhibited neuronal and oligodendrocyte differentiation, and promoted astrocyte differentiation through Noggin over-expression (Tsan et al. 2016). These studies demonstrate roles for different miRNAs in SVZ neurogenesis; more work identifying other miRNAs involved in the regulation of adult neurogenesis may provide new insight into the choice between NPC self-renewal and differentiation. Further elucidation of

miRNA function may also identify novel approaches to identify possible treatment of CNS injury and cancers.

5.1.2 The structure and cellular circuit in the OB

Both periglomerular cell (PGC) and GC interneurons are continually regenerated throughout life. Most neuroblasts that reach the OB become GCs (~94%), and only 4% form PGCs or astrocytes. Migrating progenitors in the rostral migratory stream reach the OB where they differentiate into inhibitory interneurons within their targeted layers, granule cell layer GCL and glomerular layer GL, and produce neuronal activity (Lledo & Valley 2016; Lledo et al. 2008). GCs send dendrodendritic synapses to the external plexiform layer (EPL), and send projections to the EPL mitral cells and tufted cells (Lledo et al. 2008). In contrast, PGCs are located within the glomerular layer and can synapse with cells within a single glomerulus or from different glomeruli. PGCs can be classified into three subtypes based on their expression of tyrosine hydroxylase (TH), Calretinin or Calbindin (Parrish-Aungst et al. 2007). TH+ and Calbindin+ cells within the PGC layer are mainly expressed during embryogenesis and decline postnatally, whilst Calretinin+ GC and PGC generation increases during the adult stage (Batista-Brito et al. 2008). Different regions of adult SVZ are shown to be associated with the generation of GCs and different types of interneurons. For example, dorsal regions of the adult SVZ give rise to GCs and TH+ PGC, while ventral regions generate mostly GC and Calbindin+ PGC (Young et al. 2009).

5.1.3 Functional significance of adult SVZ neurogenesis

Adult neurogenesis in the SVZ generates neuroblasts that migrate from the SVZ in the RMS to reach the OB. In the OB about 50% of these newly-generated, differentiated, matured neurons are eliminated by apoptosis within the first 2 weeks of birth, and only about 10% survive for the next year (Winner, et al. 2002). A surge of studies in recent decades have been trying to investigate the implications of generating new neurons in the OB, because it provides a good model of investigation based on the direct response between olfactory stimuli and generation of new neurons (Lepousez et al. 2015; Lledo et al. 2006b). It is still unclear how these newly generating cells are integrated within the existing neural circuit and whether they have specific significant functions. Recent publications, which have aimed to identify the function of these newly generated neurons, have generated more confusion rather than demonstrating a clear role. The suggested roles include: diminished fear responses to conditioned odours (Valley et al. 2009); reduced short-term olfactory memory (Breton-Provencher et al. 2009); reduced long-term olfactory memory (Lazarini et al. 2009); increased survival of new-born neurons in odour-rich environments (Rochefort et al. 2002); and facilitation of learning and improved memory (Alonso et al. 2012). All these different research outcomes have further complicated the understanding of the specific role of SVZ adult neurogenesis. In one recent study (Díaz et al. 2017), ionizing radiation was used at a level high enough to deplete cell proliferation without subsequent recovery and this greatly damaged the proliferating cells during the adult stage. Surprisingly, the olfactory ability of animals was mostly not affected, although novel odorant discriminating skills were impaired in irradiated mice. This was

explained by modulation of mitral cell activity in glomerular interneurons associated with OB plasticity. Therefore, allowing the irradiation damage to be controlled, suggesting some compensatory mechanisms to overcome adult neurogenesis reduction (Díaz et al. 2017). However these research findings have been contradicted by other studies, which found disrupted synaptic function and impairment in odour discrimination skills (Breton-Provencher et al. 2009; Mouret et al. 2009; Moreno et al. 2009; Sailor et al. 2017). Therefore, development of new research approaches will be useful to reveal the functional significance of newly generated neurons in the OB. For example, new studies with the aid of advanced molecular and optical approaches to selectively target adult-born neurons may help to uncover their role. One recent approach was to use live imaging microscopy to identify adult-born granule cells (GCs) over time (up to 7 weeks). They showed how the odorant responses of individual adult-born GCs are elevated as they integrated into the olfactory circuit, and how their responsiveness is increased in an enriched environment. However, it is still unclear if this responsiveness improvement is across all odours, or only selectively for odours used in the enrichment (Wallace et al. 2017).

5.1.4 Aims

In Chapter 3, we investigated miR-21's role in the DG and found that loss of miR-21 resulted in reduced adult hippocampal neurogenesis and impaired learning. Additionally, overexpression of miR-21 increased neurogenesis which was reflected in improved learning and memory in the water maze task. This miR-21 function is associated with the regulation of NPCs survival through altered expression level of the apoptotic protein Caspase-3. The regulatory role of miR-

21 in the adult hippocampal neurogenesis prompted us to investigate the role of miR-21 *in vitro* using dissected SVZ tissue from adult mice. This can allow us to study the differences in precursor cell number and their potential, and also to address the intrinsic specification of the cells when removed from their normal external environment. Additionally, the impact of miR-21 on adult SVZ neurogenesis was also investigated *in vivo* to establish the role of miR-21 in proliferation and differentiation of NPCs.

Therefore, this chapter aims to discuss the following:

- To investigate the impact of miR-21 on the NPCs from the SVZ by assessing their proliferative ability and their differentiation into cells of the neural lineage;
- To investigate the role of miR-21 on migrating neuroblasts through the RMS in the brain, and their subsequent differentiation in the OB

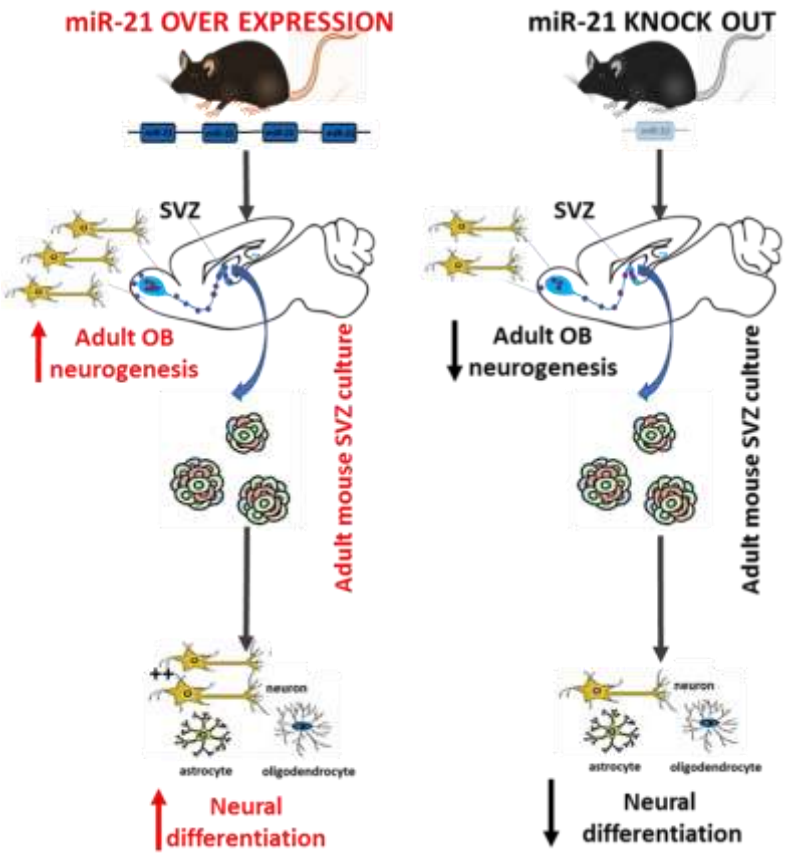


Figure 5-1: Investigating adult SVZ neurogenesis study design and results expected *in vivo* and *in vitro*.

Adult transgenic miR-21 KO mice exhibited reduced SVZ-OB adult neurogenesis, while miR-21 OE increased SVZ-OB adult neurogenesis *in vivo*. Differentiated NPCs isolated from the SVZ of miR-21 KO showed reduced neurogenesis, while the miR-21 OE NPCs had increased neurogenesis.

5.2 Results

5.2.1 Assessing *in vitro* neural differentiation of neural precursors derived from miR-21 KO and miR-21 OE mice

The effects of miR-21 on SVZ-derived adult NPCs prepared from dissected SVZ of 8-week-old adult mice were studied. NPCs from the SVZ were cultured from miR-21 knockout and wildtype mice with fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), heparin and B27, in DMEM/F12 media. The self-renewal capacity of miR-21 KO and WT culture for neural stem cells was determined by clonal analysis, which includes sphere size and sphere numbers. Neurospheres from both WT and miR-21 KO culture had self-renewal ability to generate NPCs as demonstrated by their ability to form spheres of more than 200μm in diameter, indicating no effects of miR-21 loss on proliferation capacity. Statistical analysis of neurosphere diameter from miR-21 KO compared to WT neurospheres showed no significant difference (P=0.3303, Figure 5-3A-C). Additionally, the number of neurospheres derived from miR-21 KO and WT groups was not significantly different; although the number of spheres derived from WT SVZ was higher than in the miR-21 KO, but results failed to reach significance (P=0.0831, Figure 5-3D).

Next, neurospheres were dissociated and cultured for another 7 days without growth factors to enable differentiation into the neural cell types. BrdU was added on day 6 to label proliferating cells, and on day 9, differentiated cells on coverslips were fixed, stained and analysed. Immunostaining with BrdU and quantification for BrdU+ cells showed no significant difference between miR-21 KO and WT groups (P=0.1774, Figure 5-3E). Additionally, immunostaining for the NPC

marker, Nestin, and cell quantification showed no significant difference between miR-21 KO and WT groups (P= 0.4322, Figure 5-3F), indicating that miR-21 loss does not affect NPC proliferation, which was consistent with our adult hippocampal neurogenesis *in vivo* results.

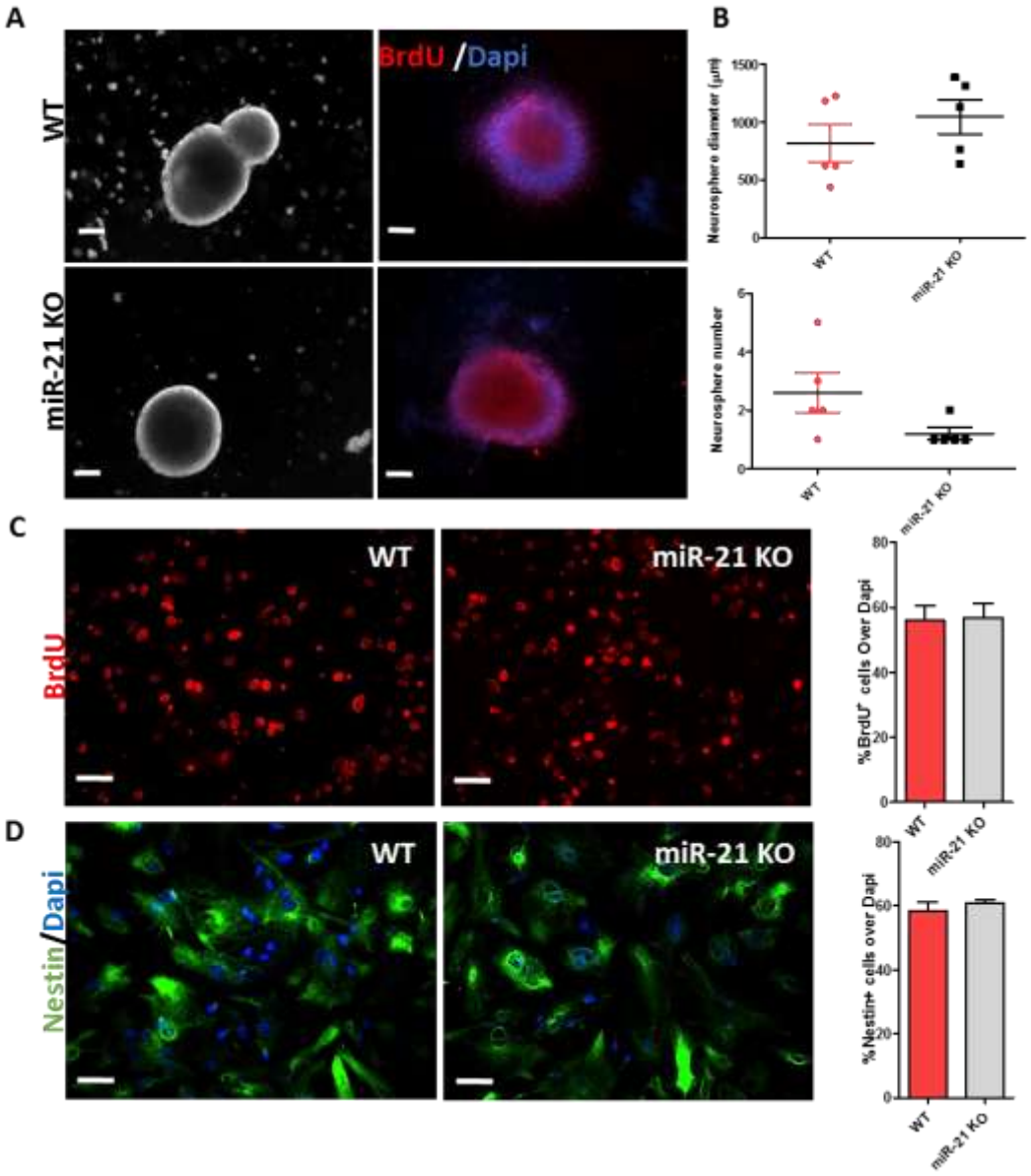


Figure 5-2: Loss of miR-21 did not affect NPC proliferation.

(A) Bright field images and immunofluorescence images for neurospheres from WT and miR-21-KO. Scale bar=200 µm. **(B)** Graphs represent the diameter and number of spheres respectively, showing no significance between the two groups. **(C)** Representative immunofluorescence images showing proliferating cells (BrdU) from WT and miR-21 KO differentiated SVZ cultures. Graph represents the percentage of BrdU+ cells over Dapi cells. **(D)** Representative immunofluorescence images showing NPCs (Nestin+) cells from WT and miR-21 KO differentiated SVZ cultures. Graph represents the percentage of Nestin+ cells over Dapi+ cells. Scale bars = 50 µm. Graphs represent the mean of three coverslips from each treatment. Values are plotted as means ±SEM.

To further assess the capacity of miR-21 to influence the differentiation of SVZ cells, *in vitro* differentiation assays were conducted. The use of growth factors including FGF and EGF *in vitro* causes neural stem cells to proliferate and maintain an undifferentiated state, while their absence allows neural stem cells to differentiate (Reynolds & Weiss 1992). Therefore, neurospheres were dissociated and plated with no mitogenic EGF or bFGF. BrdU was added 72 hours prior to plating on Poly-D-lysine (PDL) coated coverslips. On day 17, cells were fixed using 4% PFA. To measure the formation of neurons, astrocytes and oligodendrocytes, the differentiated NPCs were stained with Tuj1, GFAP and NG2 respectively.

MiR-21 KO cultures showed about 40% decrease in Tuj1+ cells compared to cultures derived from WT animals (P=0.0016, Figure 5-4A, D). However, there were no significant differences in the number of GFAP+ and NG+ cells (Figure 5-4B-D). This suggests that loss of miR-21 resulted in decreased generation of neurons in culture, but miR-21 is not essential for basal SVZ-NPC differentiation into a neuronal lineage, as they are multipotent and able to generate all linages. These results demonstrate that the overall effect of miR-21 *in vitro* is to affect the differentiation and/or survival of NPCs.

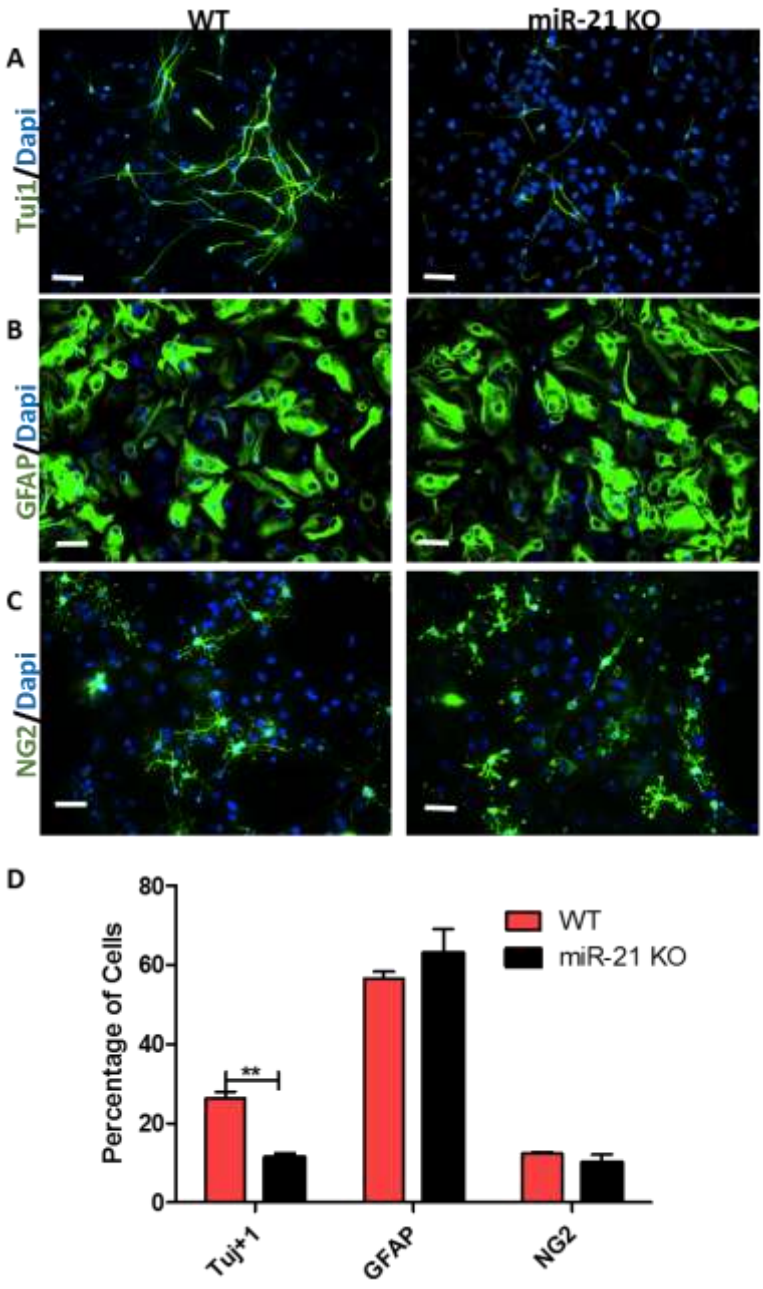


Figure 5-3: Effect of miR-21 on adult SVZ-NPCs neuron production.

(A) Neurosphere cells differentiate into neurons, as shown by Tuj1, **(B)** astrocytes as shown by GFAP staining, **(C)** and oligodendrocytes as shown by NG2 staining. Scale bars: 50 µm. **(D)** Quantification of the percentage of neurons, astrocytes and oligodendrocytes generated in WT and miR-21 KO cultures. The graph shows a significant decrease in differentiated neurons in miR-21 KO culture. Statistics were calculated using two-way ANOVA. Graphs represent the mean of three coverslips from each animal group and values were plotted as means ± SEM. **P<0.01.

As shown in the previous result, loss of miR-21 resulted in a reduction in NPC differentiation towards a neural fate. Further analysis of neurons derived from miR-21 KO SVZ neurospheres revealed that miR-21 KO-derived neurons were characterised by shorter neurites compared to WT neurons (Figure 5-5 A-B), with an average neurite length of 94.73µm compared to 62.83µm for WT neurons. This suggests that miR-21 has a role in modulating neurite outgrowth, and this may possibly be a regenerative role. Additionally, we wanted to investigate whether overexpression of miR-21 will result in increased neuronal lineage compared to WT. Quantification of Tuj1+ cells in culture derived from the SVZ of miR-21 OE animals showed no increase in neuron numbers compared to WT ($p=0.2033$, Figure 5-5 C-D).

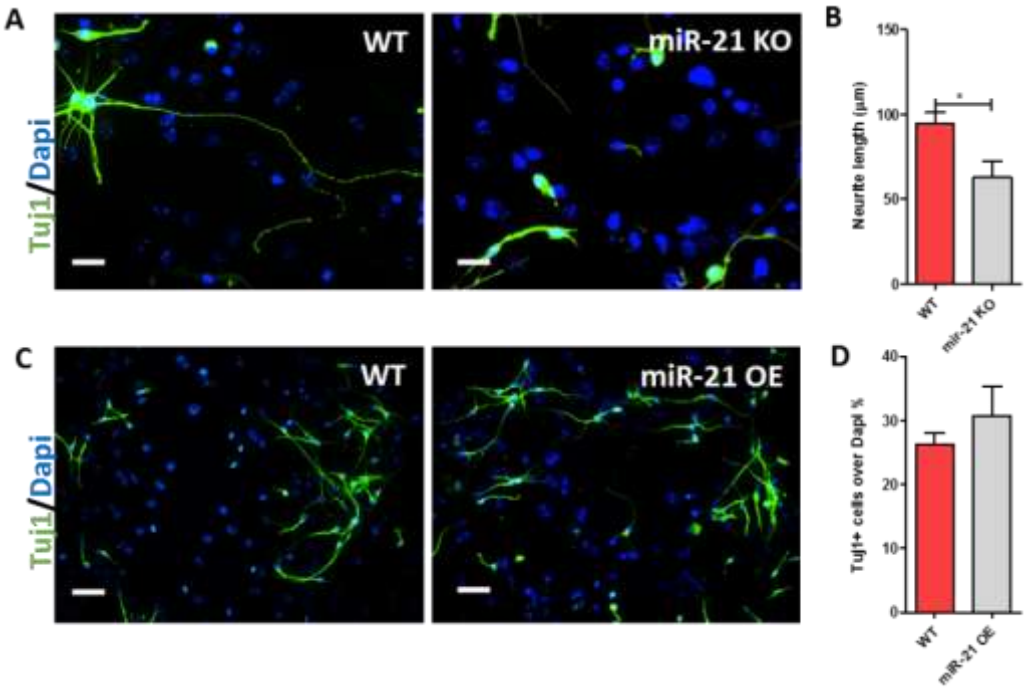


Figure 5-4: miR-21 knock out reduces neurite outgrowth.

(A) Immunofluorescence images for neurons from WT and miR-21 KO, showing reduced neurite outgrowth when miR-21 was absent. Scale bar= 20µm **(B)** Neurites from each group were characterised and counted from 3 coverslips per group. Significant reduction in neurite outgrowth was detected in miR-21 KO cells (P=0.0465, Student's t-test). **(C)** Immunofluorescence images for WT and miR-21 OE differentiated neurons. **(D)** Analysis for neuron quantification showed no significant differences between WT and miR-21 OE cultures. Data are presented as the mean from each group ± SEM.

5.2.2 Reduced neurogenesis in the OB of miR-21 KO mice

Given the effect of miR-21 loss on neuronal differentiation in adult SVZ-NPCs *in vitro*, we wanted to investigate whether miR-21 has a role in neurogenesis in the adult SVZ *in vivo*, specifically if miR-21 has any impact on glia-like cell (type B cell) proliferation in the area.

BrdU injection to label actively dividing cells and quantification of BrdU+ cells in the SVZ can indicate if miR-21 has effects on proliferation (Figure 5-6A). Statistical analysis using one-way ANOVA showed no significant differences in the number of BrdU+ cells in miR-21 OE and miR-21 KO animals relative to WT animals ($F_{(2,6)}$= 0.1809, P= 0.8389, Figure 5-6B-C). This result was further confirmed by using another endogenous proliferation marker Ki67; no significant differences were detected in the number of Ki67+ cells in the SVZ between all three groups of animals ($F_{(2,6)}$= 3.148, P= 0.1162, Figure 5-6D-E). Thus, this indicates that miR-21 has no effects on proliferation in the SVZ; these results are consistent with the results obtained in Chapter 3 (miR-21 role in proliferation in the DG).

In the SVZ, ependymal cells give rise to glia-like cells of quiescent B cells that when activated, give rise to rapidly dividing type C cells which in turn divide several times before they become neuroblasts (type A cells), which migrate and differentiate in the OB. Because miR-21 has no effects on proliferation in the SVZ, we anticipated that it may involve in regulating NPC survival. Therefore, in order to investigate the effect of miR-21 loss on neuronal precursor cells in the SVZ, we stained for late type C cells and type A cells using the DCX marker. There was a slight increase in DCX labelling in the SVZ of WT animals compared to miR-21

KO animals as determined by the area of intensity, but this did not reach significance ($F_{(2,6)}$=8.105, P=1.452, Figure 5-6F-G). Similarly, there was an increase in the DCX labelled area of in miR-21 OE SVZ compared to the WT, but again results failed to reach significance.

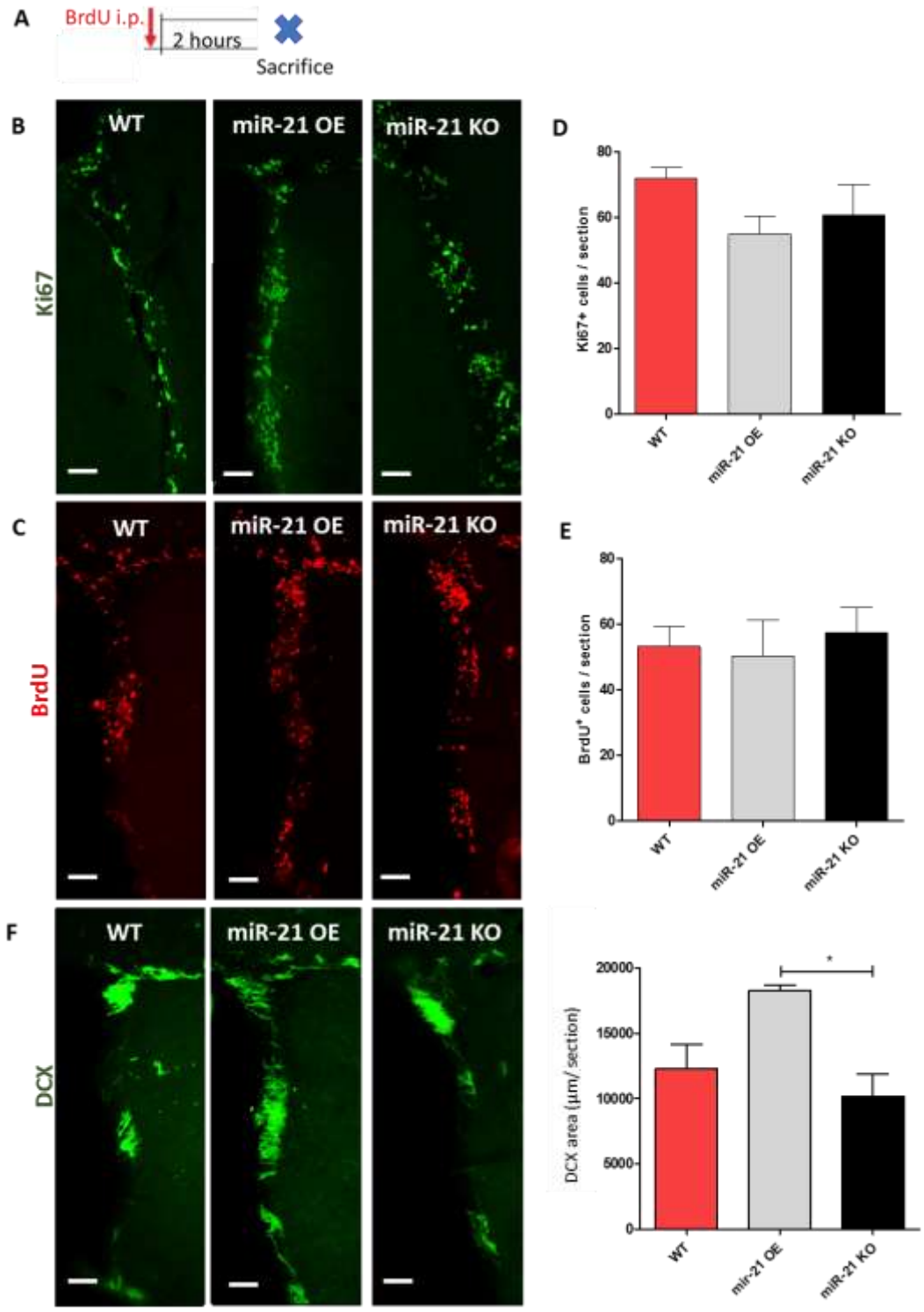


Figure 5-5: miR-21 does not affect proliferation in the SVZ.

(A) The schematic of the experiment. **(B&C)** Representative immunofluorescence images for WT, miR-21 OE and miR-21 KO, labelled with BrdU and Ki67 respectively (n=3).

Scale bar=100μm. **(D-E)** Graphs showing no differences in the number of BrdU and Ki-67 +cells in the SVZ of the different groups. **(F)** Representative immunofluorescence images for WT, miR-21 OE and miR-21 KO stained with immature neuronal marker (DCX). **(D)** Graph showing reduced volume of DCX positive area in the SVZ (miR-21 OE Vs miR-21 KO, P= 0.0197). Scale bar= 100μm, n= (3). Results were analysed with one-way ANOVA, values plotted as means +/- SEM.

The adult RMS comprises organized chains of migrating neuroblasts which migrate a relatively long distance (5-8 mm) to the OB, their target area. When migrating cells reach the OB they detach from the migratory chain and migrate radially to the OB. Around day 2 to day 7 after their birth in the SVZ, immature neuroblasts start migrating along the RMS to the OB (Carleton et al. 2003). In order to determine whether miR-21 affects migration and survival of NPCs that migrate along the RMS, mice received 3 intraperitoneal injections of BrdU every two hours to label adult born NPCs and animals were sacrificed at 3 days after BrdU injection (Figure 5-7A). Consistent with the observations in the *in vitro* SVZ culture experiments, quantification showed no significant differences in the number of proliferating BrdU+ cells between miR-21 OE mice compared to WT mice and between miR-21 KO mice compared to WT mice ($F_{(2,6)}$ =0.9223, $F_{(2,8)}$=0.9413, Figure 5-7B, F). Similar observations were recorded for Ki67+ assessments (Figure 5-7C, G) thus indicating that miR-21 is not involved in the regulation of the rate of adult NPCs proliferation in the SVZ. Neuroblasts migrate from the SVZ along the RMS in chains or tube-like structures in close proximity to multipotent GFAP+ astrocytes, which maintain the RMS structure. Therefore, we wanted to investigate if the astrocyte migration pattern differed in miR-21 OE and miR-21 KO compared to WT animals. GFAP+ cells in the RMS showed similar migration patterns in all animal groups indicating that miR-21 has no effect on astrocyte migration (Figure 5-7D).

Next, migrating neuroblasts in the RMS were measured to assess the effect of miR-21 on precursor neuron migration. DCX marker was used to label migrating neuroblast, and measurement of the volume of DCX+ cells in the RMS showed a

significant reduction in miR-21 KO compared to the WT animals ($F_{(2,6)}$ = 9.339, p= 0.0144, Fig 5-7E, H).

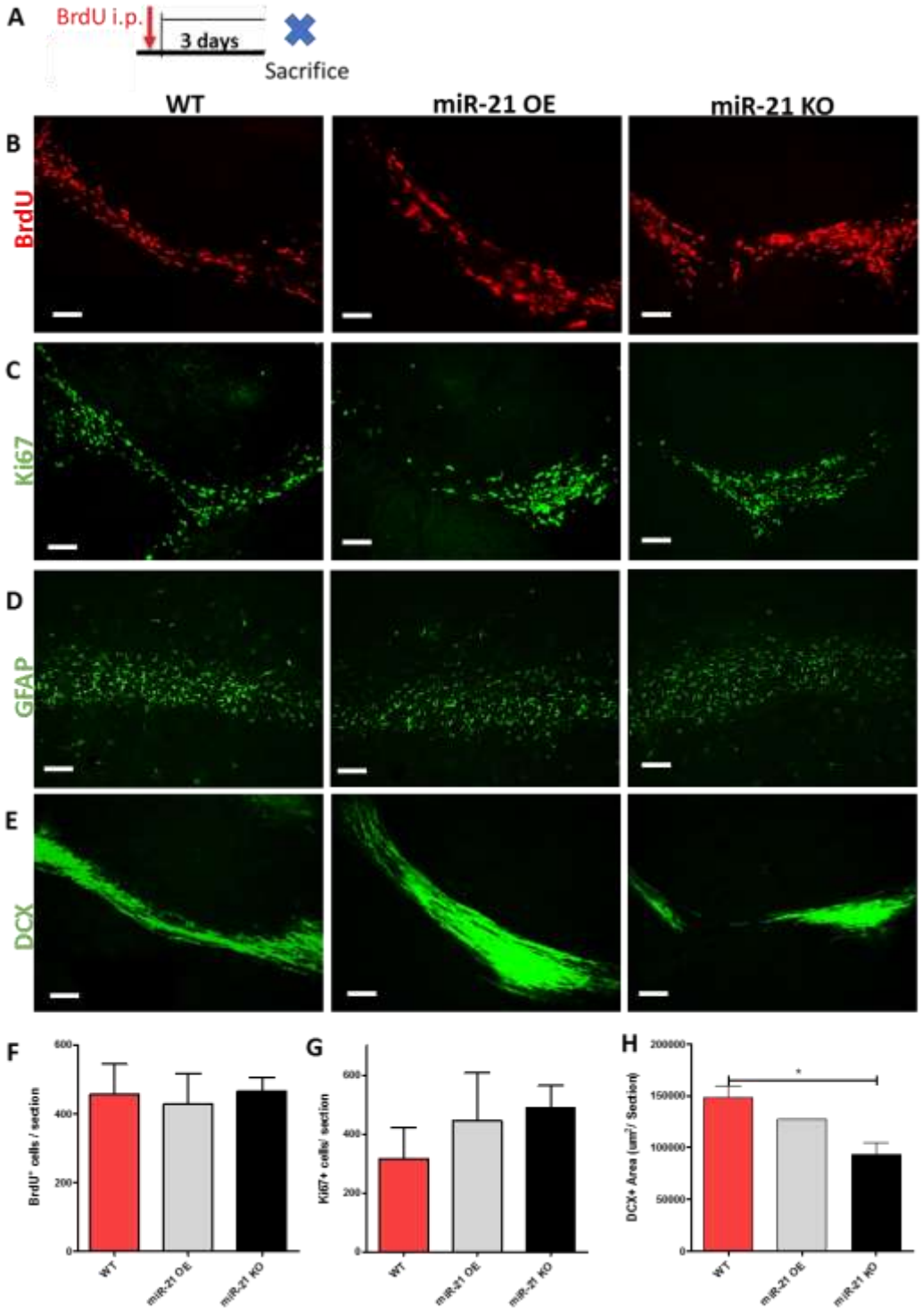


Figure 5-6: Reduced migrating progenitors in miR-21 KO RMS.

(A) Shows experimental timeline. **(B-C)** Representative immunofluorescence images for WT, miR-21 OE and miR-21 KO respectively, labelled with Ki67 and BrdU. **(D)** Immunofluorescence images for WT, miR-21 OE and miR-21 KO labelled for GFAP. **(E)** Immunofluorescence images stained with immature neuronal marker (DCX), showing reduction in DCX+ cells in miR-21 KO SVZ. Scale bar=100μm. **(F)** Graphs showing no change among groups in the number of BrdU and Ki-67+ cells in the SVZ. **(G)** Graph showing reduced volume of DCX + area in the SVZ (WT Vs miR-21 KO, P=0.0144). n= (3). Results were analysed with one-way ANOVA, values plotted as means ±SEM.

When migrating neuroblasts reach the OB, they detach from the migratory chain and migrate radially and then differentiate into two types of different interneurons. Most cells (95%) differentiate into GABAergic granule neurons and contribute to the neuronal population in the granule cell layer (GCL), which can be detected by (NeuN) neuronal marker, and the remaining (~4%) add to the glomerular layer and express dopamine and GABA neurotransmitters, which can be detected by tyrosine hydroxylase (TH) (Bonzano et al. 2016). Calretinin is one of the calcium binding proteins that is expressed in the OB and have been detected in different neuronal populations within the GCL and GL, allowing the identification of cytoarchitecture differences among different transgenic mice groups compared to WT animals (Wu et al. 2017). Based on the obtained results of reduced migrating precursor cells in the RMS of miR-21 KO mice, we hypothesised that the size of OB may be reduced. Therefore, to investigate this hypothesis, the radii of OBs from different groups were measured and analysed (Figure 5-8A). Statistical analysis showed no significant differences in the radius of the OB of miR-21 OE or miR-21 KO mice compared to WT mice (Figure 5-8B, E). In addition, the thickness of the GCL and GL layers did not show any significant differences between different groups ($F_{(2,6)}$=0.2450, P= 0.7902, $F_{(2,6)}$=0.7196, P= 0.7196, Figure 5-8B, E). Similarly, the OB whole size and different OB layers were measured and analysed. However, results showed no significant differences in the size of different layers (GCL and GL respectively) in miR-21 OE and miR-21 KO animals compared to the WT animals ($F_{(2,6)}$=1.110, P=0.3890, $F_{(2,6)}$= 2.805, P=0.138, Figure 5-8D, F). To determine any general changes in specific OB interneuron subpopulations and detect the cytoarchitecture in miR-21 KO and

miR-21 OE animals, we also performed immunohistochemistry with the Calretinin marker and found no differences in Calretinin+ cell distribution in the different groups (Figure 5-8C). This therefore indicates that reduced migratory neuroblasts was not reflected in the total OB size or OB layers.

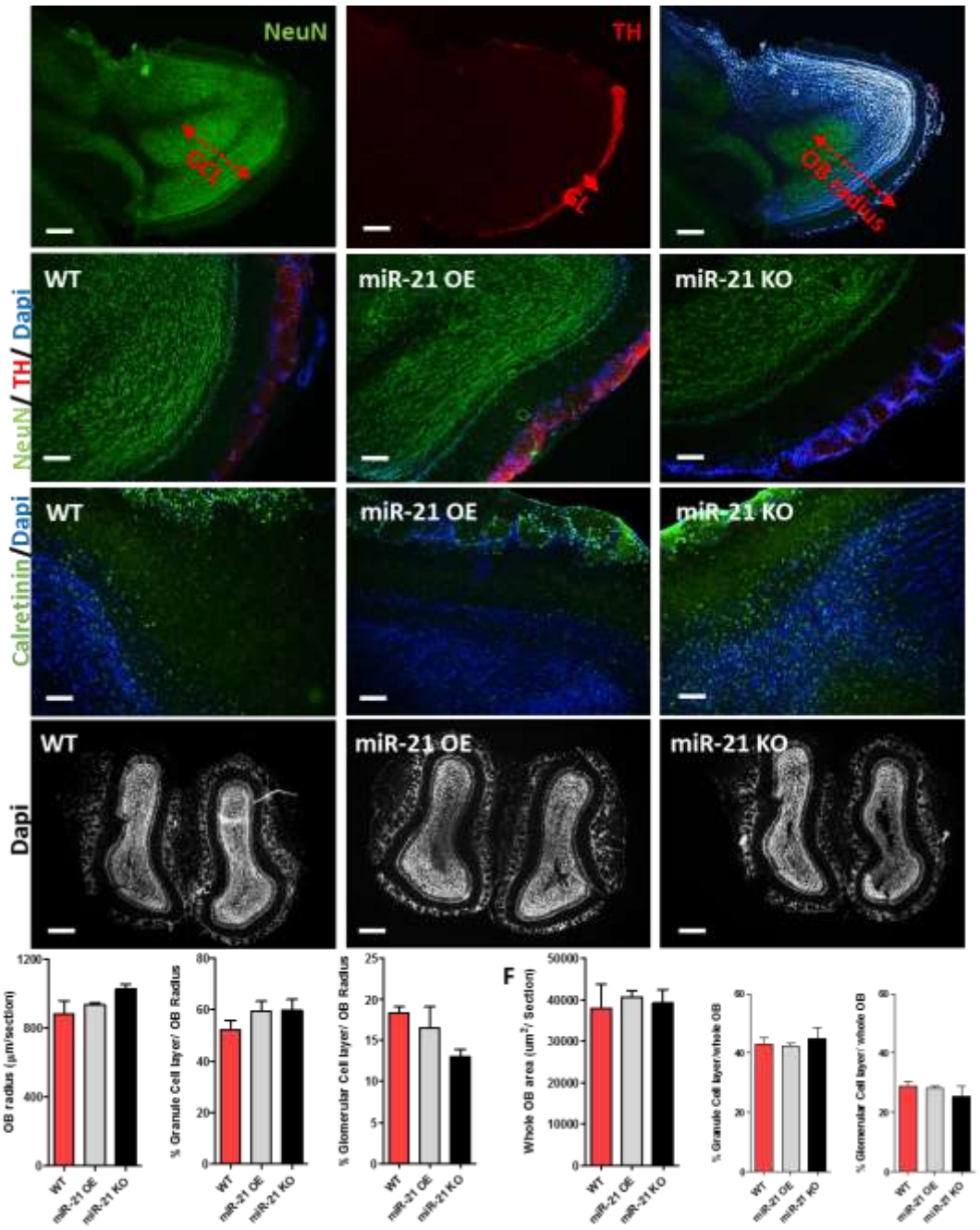


Figure 5-7: Reduced progenitors' migration in the RMS of miR-21 KO mice does not affect absolute size of OB.

(A) Representative images showing measurements of the GCL and GL in relation to the whole OB radius. Scale bar= 200 µm. **(B)** Representative images from each group showing different OB layers immunostained with NeuN for GCL and TH for GL; miR-21 KO neuroblasts able to differentiate into NeuN+, TH+. **(C)** Representative images from each group showing Calretinin+ cells in the GCL and GL; no differences were detected in the distribution of Calretinin+ cells in the different groups. Scale bar= 50 µm. **(D)** Representative images from different groups stained with nuclear staining (Dapi), indicating similar OB size among different groups. Scale bar=200µm. **(E-F)** Graph represents measurement of the GCL and GL relative to the whole OB size in WT, miR-21 OE and miR-21 KO animals. (n=3). Results were analysed with one-way ANOVA to determine statistical significance and values were plotted as means +/- SEM.

Although we observed reduced neuroblast migration in RMS of miR-21 KO brains, this decrease was not reflected in a reduced total OB size or GCL and GL within the OB. It is possible that the loss of miR-21 may influence the size of differentiated neurons without affecting overall bulb size, or these adult newly generated neurons are going through apoptosis; thus, reduced neuroblast migration associated with miR-21 inhibition was not reflected in the bulb size.

To address this possibility, we next quantified the number of mature neurons in the GCL. The majority of migrated neuroblasts (94%) that reach the OB differentiate into inhibitory GCs within the GCL. Additionally, 40% of the GCs in the adult OB are generated postnatally that replace the pre-existing GCs produced during neonatal stages (Imayoshi et al. 2008), thus measurement of the inhibitory neurons in the GCL would be sufficient to assess for the effect of miR-21 on OB neurogenesis. Immature neuroblasts migrate radially in the OB and differentiate about 3 weeks after their birth to integrate with the neural population in the GCL and GL; this can change olfactory behaviour and circuits (Pignatelli & Belluzzi 2010). These newborn neurons acquire mature morphology and start to have synaptic connections and express mature neuronal marker NeuN (Petreanu & Alvarez-Buylla 2002). Thus, the number of NeuN+ cells in the GCL was quantified. MiR-21 KO mice had similar numbers of NeuN+ cells in the GCL, compared to the WT and miR-21 OE animals ($F_{(2,6)}$ =1.993, P=0.2169, Figure 5-9C, E). This indicates that miR-21 loss and over expression did not affect the total population of mature neurons in the OB.

One major caveat to the previous assessments is that the effects of any impaired or increased neurogenesis in the miR-21 KO or miR-21 OE mice respectively are too subtle to affect a discernible phenotype in measures such as neuronal number or OB size. In order to examine specifically the number of adult newborn neurons in the OB, long-term BrdU administration was used. Mice received ip. injection of BrdU for five continuous days and were allowed to survive for an additional 3 weeks to give enough time for all BrdU labelled cells to exit the RMS and reach either the GCL or GL (Wojtowicz & Kee 2006) (Figure 5-9C). Quantification for BrdU+NeuN+ cells in the GCL of the OB showed a significant reduction in miR-21 KO mice compared to the WT animals (P=0.0228, Figure 5-9D, F). These BrdU+ cells were also double stained with NeuN marker for mature neurons to validate that they were neurons. This result indicates that although loss of miR-21 did not affect the total neuronal population in the OB, it reduced the number of adult newborn neurons in the same area. Whether this reduction is sufficient to introduce functional significance in olfactory function remains to be determined.

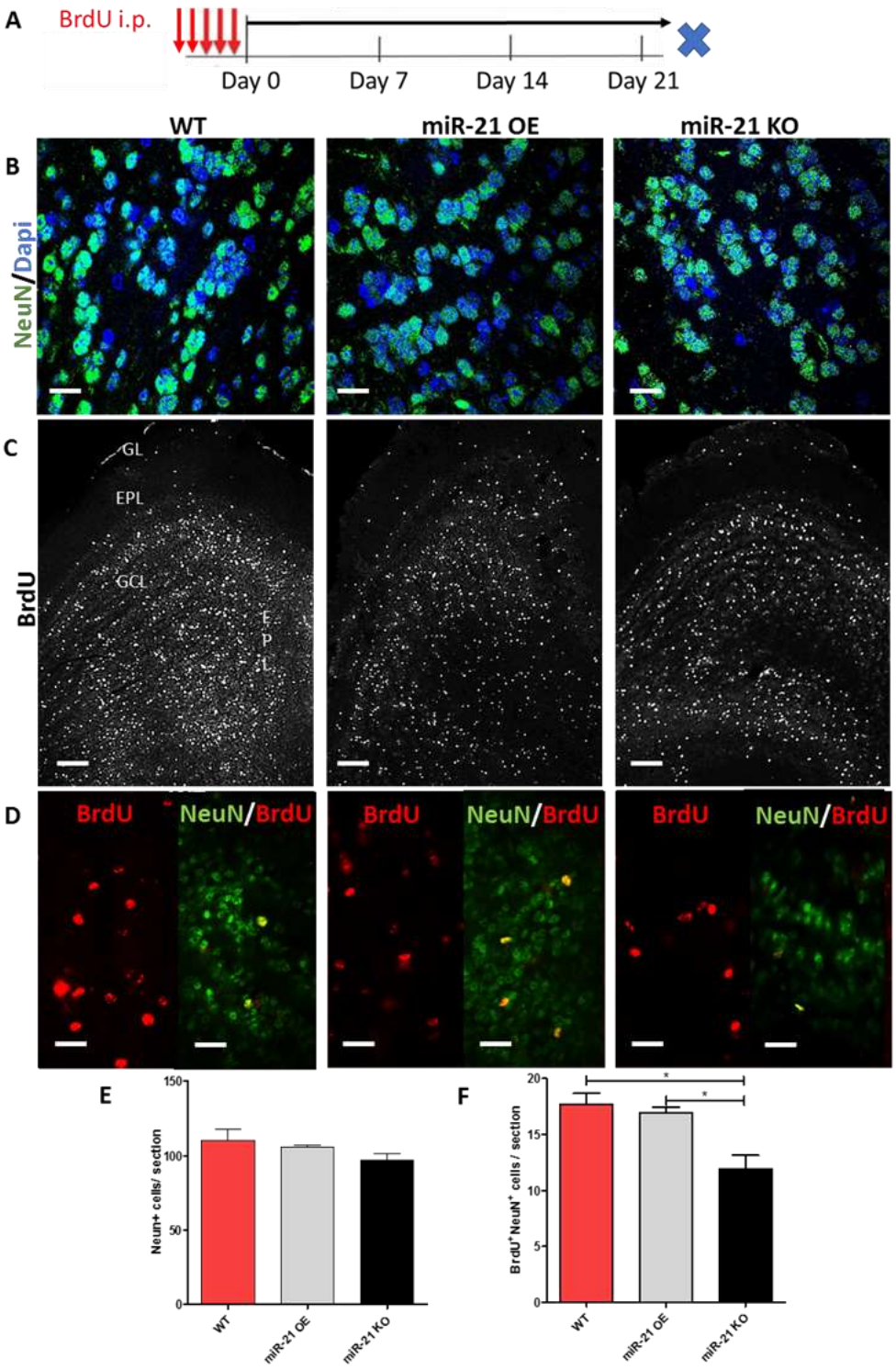

A
BrdU i.p.
Day 0
Day 7
Day 14
Day 21
WT
miR-21 OE
miR-21 KO
B
NeuN/Dapi
C
BrdU
GL
EPL
GCL
E
P
L
D
BrdU
NeuN/BrdU
E
Neun+ cells/ section
150
100
50
0
WT
miR-21 OE
miR-21 KO
F
BrdU+NeuN+ cells / section
20
15
10
5
0
*
*
WT
miR-21 OE
miR-21 KO

Figure 5-8: miR-21 inhibition affects adult newborn neuroblast differentiation in the OB.

(A) Schematic illustrating the experimental timeline. **(B)** Representative images showing BrdU+ neurons in the granule cell layer (GCL), external plexiform layer (EPL), and glomerular layer (GL). Scale bar=100 µm. **(C)** Confocal images illustrating adult new-born NeuN+ cells in WT, miR-21 OE and miR-21 KO mice. Scale bar=10 µm. **(D)** Confocal images represent BrdU+NeuN+ neurons in the (GCL), miR-21 loss led to reduced neurogenesis in the GCL ($p < 0.05$). Scale bar=15 µm. **(E)** No significant difference in the NeuN+ cells quantification in the GCL between the different groups. **(F)** Graphs illustrating the numbers of BrdU+NeuN+ cells in the OB of miR-21 KO animals and WT mice ($p<0.0109$). Results were analysed with one-way ANOVA to determine statistical significance and values were plotted as means ± SEM.

5.2.3 Neurogenesis in the SVZ is not impaired at postnatal day 30

Transgenic mice used in this study were generated with a global KO and a global over-expression of miR-21. To check that the observations described in the previous sections were not due to developmental effects, specifically the development of the OB structure, we examined brains at an earlier developmental stage (P30). NPC characterisation in the SVZ and RMS was performed using the DCX marker. There was no significant difference in DCX staining in the WT, miR-21 OE and miR-21 KO animals in the SVZ ($F_{(2,6)}$=1.102, P=0.3913, Figure 5.10) and in the RMS ($F_{(2,6)}$= 1.191, P=0.3667, Figure 5.10). This indicates that results obtained in this study are due to miR-21 effects in the adult stage and not due to developmental regulation in miR-21 transgenic animals.

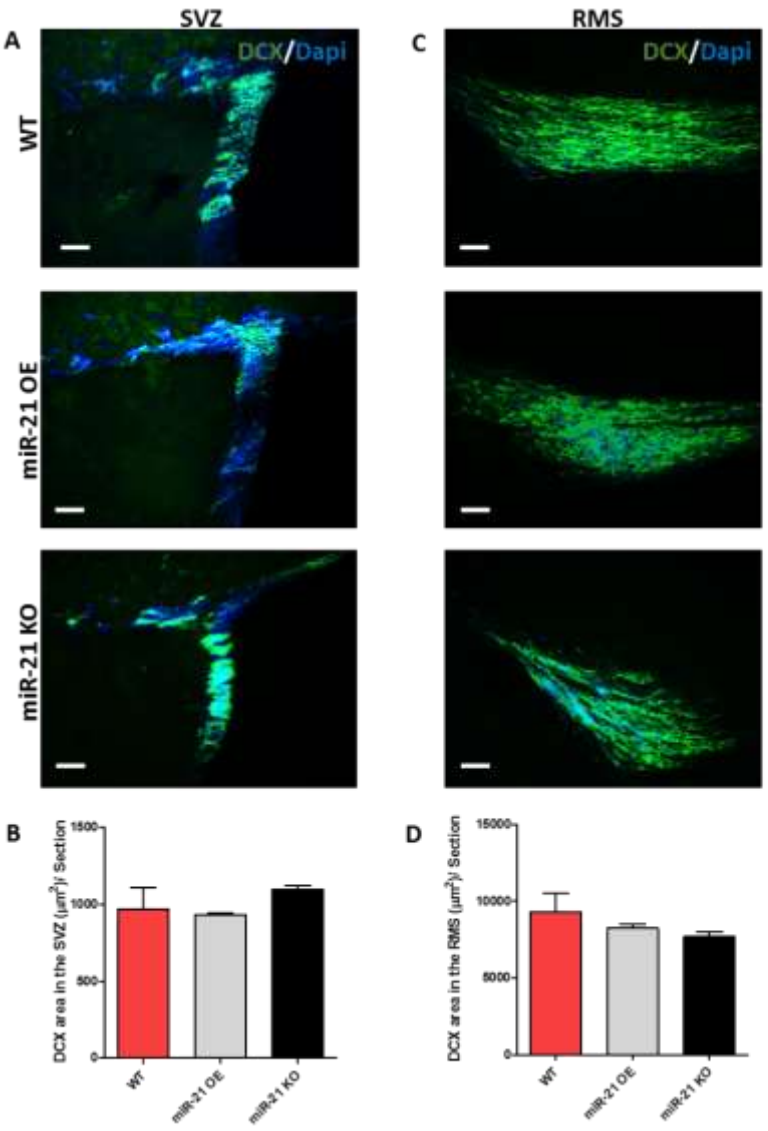


Figure 5-9: Neurogenesis in the SVZ and RMS at postnatal day 30.

(A) Representative images to demonstrate DCX+ staining in the SVZ in WT, miR-21 OE and miR-21 KO animals **(B)** No significant differences in the measurement of the area of the SVZ in the three groups. (C) Representative images for the RMS in WT, miR-21 OE and miR-21 KO animals. (n=3). Scale bar = 100µm. **(D)** No significant differences in the measurement of the area of the SVZ in the three groups. Results were analysed with one-way ANOVA, values were plotted as means +/- SEM.

5.2.4 miR-21 loss does not influence neurogenesis in the SVZ of mice aged 12 months

Reduction in adult neurogenesis is a common feature in several neurodegenerative diseases including AD, PD and HD and different animal models of neurodegenerative diseases have shown impaired adult neurogenesis, which can be linked to the loss of neuronal cells (Winner & Winkler 2015). Previous reports have found links/associations between altered miR-21 expression and age, as miR-21 is developmentally regulated and its expression is reduced after early developmental stages (Olivieri et al. 2012). Investigation of hippocampal adult neurogenesis in aged compared to adult miR-21 transgenic animals indicated significant reduction in hippocampal adult neurogenesis in aged animals compared to adult animals (Chapter3, Figure 3-11C). We have seen that loss of miR-21 reduced adult born mature neurons in the OB, therefore, we wanted to investigate the effect of miR-21 on adult neurogenesis in the SVZ-OB axis in older mice (12 months). Increased miR-21 expression may act to restore its regulation in older mice and have a therapeutic effect, which can be used to stimulate neurogenesis in neurodegenerative diseases.

We immunostained for immature neurons in the SVZ and RMS using precursor marker DCX. However, quantification for DCX+ cells in both areas showed no significant differences among different groups of animals (Figure 5-11). This result indicated no role for miR-21 in the regulation of neurogenesis in older animals in the SVZ area.

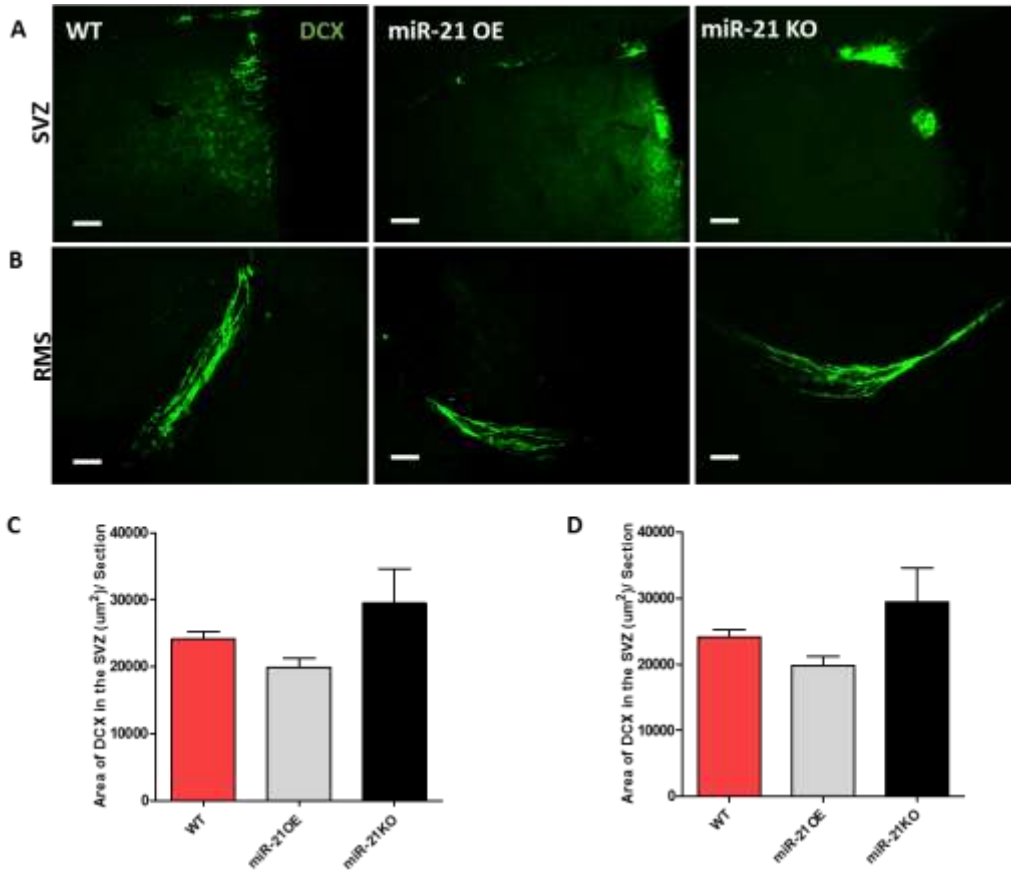


Figure 5-10: miR-21 inhibition does not influence neurogenesis in the SVZ in mice aged 12 months.

Immunofluorescence images indicated no differences in the DCX staining between the three groups. **(A-C)** Shows representative images for the SVZ, RMS and OB in WT, miR-21 OE and miR-21 KO animals (n=3). Scale bar = 100µm. **(C-D)** Figures represent no significant difference in the volume of DCX+ cells in the SVZ and RMS respectively, of miR-21 OE and miR-21 KO animals compared to WT animals. Results were analysed with one-way ANOVA, values were plotted as means ± SEM.

5.3 Discussion

We have identified that the loss of a miRNA, miR-21, can result in a shift in adult neural progenitor cell differentiation. NPCs from the SVZ of miR-21 KO mice showed reduced neural differentiation *in vitro* and *in vivo.* Furthermore, the number of adult-born differentiated new neurons were reduced in the OB of miR-21 KO mice compared to their WT littermates.

In vitro experiments were performed to see if the *in vivo* observations we have seen in the DG neurogenesis could be recapitulated in culture. Stem cells from all ages of the CNS can be stimulated to proliferate when they are exposed to growth factors in tissue culture. When appropriate plating techniques are established for the culture, continued cell division generates non-adherent spheres of cells (neurospheres). The neurosphere assay is a good technique for isolating neural stem cells and progenitor cells to investigate the differentiation and potential of cell lineages. NPCs from the SVZ of both miR-21 OE and miR-21 KO mice were able to exit the cell cycle and generate cells of all neural linages including neurons, astrocytes and oligodendrocytes. However, loss of miR-21 resulted in reduced neuronal cells (as indicated by Tuj1+ staining) in a differentiated culture derived from adult SVZ (Figure 5-4A, D). Moreover, the number of Tuj1+cells increased in miR-21 OE culture, which further suggests that miR-21 promotes neuronal differentiation, or alternatively the survival of neuronal precursors. In addition, measurement of neurite outgrowth showed a significant change in neurite length in miR-21 KO differentiated neurons as they had shorter neurites (Figure 5-5C-D). Neurite outgrowth occurs through a number of interconnected cellular processes, including protein trafficking and intracellular signalling pathways to reorganise

the cytoskeleton of the neurite (Yoshimura et al. 2006; Ditlevsen et al. 2008). miR-21 over expression *in vitro* was found to increase neurite outgrowth in adult rat dorsal rat ganglion DRG neurons and to increase the length of the neurite compared to the control (Strickland et al. 2011). This indicates possible miR-21 regulatory mechanisms underlying neurite growth, and in the case of its loss, this process is disrupted.

To investigate the role of miR-21 in adult neurogenesis in the SVZ *in vivo*, we first looked at the effect of miR-21 on proliferation. In the SVZ, type B cells give rise to type C cells which, in turn, divide several times before they become neuroblasts (type A cells), which then, within the next 3-7 days, migrate through the rostral migratory stream to the OB. Approximately 30 000 to 80 000 cells are added to the OB daily through the RMS (Abrous et al. 2005). We found no difference between NPCs proliferation in miR-21 KO and miR-21 OE compared to WT in the SVZ using two proliferation proteins, BrdU and Ki67, which was consistent with our observations in the adult hippocampus. A similar result was observed *in vitro*, as measurement of neurosphere size, which can be indicative of proliferating NPCs, was not significantly changed with miR-21 loss compared to WT neurospheres (Figure 5-3A-D).

Based on our results related to miR-21's role in hippocampal adult neurogenesis, it was predicted that miR-21 would cause more neuronal survival due to reduced apoptosis and the neuroprotective role of miR-21. In adult miR-21 KO SVZ, reduced number of late transient amplifying cells (type C) and neuroblasts (type A) was observed in miR-21 KO compared to WT mice, as indicated by the DCX

marker (Figure 5-6F, G). However, since DCX is a marker for both neuroblasts and late type C cells, another marker is needed to specify which types of cell are influenced by miR-21 and validate our DCX quantification results. Distal-less homeobox 2 protein (Dlx2) is a marker for specific to early neuronal commitment and some immature neurons in the adult brain which can be used to further validate the DCX results and selectively identify the effect of miR-21on type C cells (Naser et al. 2016).

Several reports have suggested a role for miR-21 in the regulation of cell migration in various cancer types, and, after TBI, miR-21 may also have a role in the regulation of a number of genes including *PTEN, Reck, Bcl-2, TIAM* and *PDCD4* (Ge et al., 2014; Liao et al. 2016; Xu et al., 2014; YAN et al., 2016). Nevertheless, the majority of miR-21 KO neuroblasts in the adult SVZ/RMS exited the cell cycle and migrated rostrally to the OB where they differentiated into adult OB neurons (Figure 5-7D). DCX is a marker for late transient amplifying cells, neuroblasts and immature neurons. Thus, we followed the migration of neuroblasts along the RMS. It has been reported that WT neuroblasts migrate at a speed of 106±7 μm/hour, and that its migration pattern and speed are regulated by epidermal growth factor receptor (EGFR) (Kim et al. 2009; Comte et al. 2011). Since miR-21 expression is regulated by EGFR in lung cancer patients, we wanted to investigate the role of miR-21 in neuroblast migration (Krichevsky & Gabriely 2009; Seike et al. 2009). Although we have not addressed the speed of migration, the volume of DCX+ cells in the RMS was measured and the RMS volume was found to be significantly reduced in miR-21 KO mice relative to WT mice (Figure 5-7E, G). Reduction in migrating neuroblasts in miR-21 KO RMS was expected

because reduced DCX+ volume was detected in miR-21 KO SVZ. However, to examine the role of miR-21 in migration in more detail, real-time analyses of migrating neuroblasts is needed by two-photon time-lapse microscopy (Wallace et al. 2017). Additionally, we looked at glial migration and distribution along the RMS, as it has been suggested that miR-21 regulates astrocyte migration after SCI. SCI mice with increased miR-21 expression showed a significant reduction in GFAP+ cells around the lesion site compared to WT, while miR-21 inhibition resulted in increased GFAP+ cells in the same area (Bhalala et al. 2012). GFAP+ cell present along the RMS was not changed in miR-21 OE and miR-21 KO compared to WT group, which could indicate that cell migration in the normal brain is under the regulation of other mechanisms, or that other mechanisms are compensating for miR-21 loss.

Based on our results in the hippocampus and reduced neuroblast migration in miR-21 KO adult SVZ compared to WT group, we hypothesized that the number of differentiated neurons in the OB of miR-21 KO animals will be reduced, thus affecting the whole bulb size. Therefore, we measured the whole surface area of the OB, and the GCL and GL within the bulb, in which mainly migrating neuroblasts had settled and differentiated. Analyses of the data collected indicated no significant differences between groups either for the surface area or for the measurement of GCL or GL (Figure 5-8F). Additionally, immunostaining was used to specifically differentiate cell layers, and for more accurate measurement. Again, unexpectedly, there were no significant differences in the radii of the OB, GCL and GL in miR-21 OE and miR-21 KO compared to WT group (Figure 5-8G).

Despite the continuous generation of new neurons in the adult OB, OB expansion is restricted by having controlled total number of cells, which is regulated by an accompanying mechanism of programmed cell death (Kaplan et al. 1985; Bayer 1982; Biebl et al. 2000; Ryu et al. 2016). A previous study suggested that the apoptotic mechanisms in the OB regions regulated the 0.8% - 1% addition of new adult generated neurons in the OB, which has a significant role in a self-renewal system of the adult mammalian brain (Biebl et al. 2000). This controlled neuron number within the OB is different to the increase neuronal number associated with increase neurogenesis in the adult DG (Imayoshi et al. 2008; Stanfield & Trice 1988; Bayer et al. 1982). In a study investigating the postnatal development of the OB in rats, they found that the volume of the OB at P0 to P30 increased 7-fold and the volume of the OB remained unchanged with increased age after P30. Most cellular proliferation within the GCL occurs during the first post-natal few weeks (Rosselli-Austin & Altman 1979; Petreanu & Alvarez-Buylla 2002). This suggests that the volume of different OB layers will not change due to coinciding apoptotic mechanisms even in the presence of miR-21 which promotes the migration of neuroblasts to the OB. This can be further investigated by quantification of apoptotic cells in the OB of WT mice compared to the miR-21 OE and miR-21 KO mice.

As miR-21 function might be more specific to differentiated neurons in the OB, NeuN, a marker for mature neurons, immunohistochemistry was performed. There were no differences in NeuN+ cells in the GCL of miR-21 OE and miR-21 KO OBs compared to WT OBs. This indicated that miR-21 loss does not affect total neuronal population in the adult OB (Figure 5-9E). Although a large number of

neuroblasts are added to the OB daily (Imayoshi et al. 2008; Omais et al. 2018), most of these cells do not survive, and 50% go through apoptosis the first few weeks of their arrival in the OB (Petreanu & Alvarez-Buylla 2002; Malvaut & Saghatelyan 2016). Therefore, it might be that miR-21 encourages an increase in neurogenesis, but the proportion of these newly added neurons compared to the overall neuronal number in the OB is limited due to programmed cell death activities (Biebl et al. 2000; Kim & Sun 2011). Labelling the precursors that arrive in the OB and recording the percentage of cell death in miR-21 KO mice compared to WT mice would help investigate this assumption. It could be possible that regulating cell survival in a normal adult OB is controlled by several factors and, in the case of miR-21 loss, other factors compensate for its loss. Nevertheless, this does not eliminate miR-21's effects in the adult OB as it might be involved in potential fine-tuning processes and proper integration of newborn neurons with the functional circuit in the OB, which could be investigated by looking at dendritic growth of adult born inhibitory neurons within the GCL and GL (Liu et al. 2015). However, when we looked specifically for adult newborn neurons we found reduced BrdU/NeuN+ cells in miR-21 KO adult OBs (Figure 5-9F). This result indicated that miR-21 affects the differentiation and/or the survival process of the adult newborn neurons, but it does not affect the total neuronal population in the OB. A previous study suggested a gradual increase in the number of labelled adult generated granule cells and periglomerular cells of the OB with age in normal mice. Analysis of new generated neurons in the OB over time revealed that the ratio of labelled granule cells was 40% after 6 months and 60% after 12–18 months (Imayoshi et al. 2008).

The question is whether this minor reduction in neuronal population in miR-21 KO mice will be reflected in significant OB function, or if this reduction in neuronal accumulation throughout life will have a greater impact in older animals. Therefore, long term BrdU administration may reveal if the proportion of the of BrdU labelled cells in miR-21 KO mice will significantly decrease with age, compared to WT animals. Additionally, performing behavioural OB tests may reveal if miR-21 loss will affect olfactory learning. Odour discrimination and odor exploration tasks can be used assess olfactory functions with miR-21 transgenic mice compared with WT mice. Odour exploration tasks are used to examine general olfactory functions, as animals use their odour skills and motivation to search for food-associated odours. For example, novel varieties of buried food retrieval test can be used to check if mice have a clear olfactory impairment, followed by habituation–dishabituation task to test for female intrinsic motivation to discriminate social odours (Cordero-Llana et al. 2014). However, since ablation of adult neurogenesis, which resulted in marked decrease in granule cell number in the OB did not affect odour response and odour exploration in mice in previous research, more complicated tasks are needed. Difficult odour discrimination tasks can be used to test for specific impairment in the adult born interneuron population such as discriminating the first exposure to a novel odorant with known odorants (Díaz et al. 2017).

Several studies with miRNA profiles have shown changes in miRNAs expression levels as the brain ages (Inukai et al. 2012). Additionally, age has a big impact on the onset of most neurodegenerative diseases, which is associated with

impaired disposal of excessive proteins (Abdullah et al. 2015). Also, in a previous study, identification of histological impairments in the cortex or in the CA1 and CA3 was not possible until mice reached 6 months, when the Dicer, a key endoribonuclease RNA in the biogenesis of mature miRNAs, was deleted (Cheng et al. 2014). MiR-21 has different expression levels depending on the stage of development (Olivieri et al. 2012; Põlajeva et al. 2012b). Investigations in aged animals revealed that miR-21 KO or over-expression had no effect on neurogenesis, as demonstrated by similar numbers of DCX+ cells in the SVZ and RMS area of aged miR-21 OE and miR-21 KO animals compared to WT animals (Figure 5-7). Although this result was different to what we expected, this needs to be further clarified by the measurement of total OB size in these aged animals. This will allow us to assess the impact of accumulated reduction of neurogenesis due to miR-21 loss on olfactory function. A previous report showed different miR-21 expression levels in different age groups (Olivieri et al. 2012). MiR-21 may be involved in the regulation of aging-relevant pathways including insulin signalling pathway and/or AMP-activated protein kinase (MAPK) pathways (Kubben & Misteli 2017), and indeed these pathways have been found to be regulated by miR-21 (Mei et al. 2013).

However, a number of questions remain to be answered regarding the specific function related to reduced adult neurogenesis in miR-21 KO OBs. We found a reduction in the mature adult-born neurons in the OB, however the implications on olfactory function remain to be determined. Investigation using the odour-discrimination task will enable us to determine if the limited mean reduction in GC

number in miR-21 KO OBs will lead to a behaviour deficit. Additionally, we need to understand if the loss of miR-21 will immediately affect the relationship between the activity of mature adult-born neurons and the OB circuit activity, or if the effect is going to be cumulative and become apparent with increased age. This can be achieved by studying neuronal population at different time points, which can help lead to better characterisation of the developmental process for adult new-born neurons and their engagement with the existing neural circuit.

Chapter 6 General Discussion

Adult neurogenesis research is concerned with the biology of generating neurons from NPCs in the adult brain; the insight gleaned from this research will help us understand the role of newborn neurons in normal physiological processes in the brain. In recent years there has been research uncovering the role of adult neurogenesis in learning and memory formation, especially for the formation of spatial and fear memories. This area of research is also important as our knowledge of regenerative mechanisms, particularly after brain injury or neurodegenerative diseases, is incomplete. Additionally, this knowledge can help answer fundamental questions related to stem cell regulation, fate determination, adult neuronal plasticity and the molecular mechanisms underlying the development of neuronal diseases. However, these studies have also led to some degree of conflicting results which need to be further validated and clarified. One important area that can potentially benefit from this research is the identification and development of novel therapeutic factors that can be used to treat neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). A common feature of these diseases is progressive loss of specific populations of neurons within the brain, thus molecules that contribute to increased neurogenesis can provide a means of replacing lost neurons; furthermore knowledge on how disease-related proteins act on newly generated neurons is also important in translating these new discoveries into therapeutic approaches (Winner & Winkler 2015).

In this project, the microRNA miR-21 was investigated as it is a factor that is well known to be involved in increasing cell proliferation and survival in many cancer types including glioma as well as after traumatic brain and spinal cord injury (Krichevsky & Gabriely 2009; Buscaglia & Li 2011a). This dissertation have tried to answer an important question; how do neural progenitor cells (NPC) and differentiated neurons in normal adult brain react to altered expression levels of miR-21? The study showed that miR-21 loss has a significant effect on the differentiation of NPCs and the survival of differentiated neurons. Adult miR-21 KO mice demonstrated decreased hippocampal neurogenesis as evidenced by the reduction in the number of immature and mature neurons labelled with DCX and NeuN markers respectively. This reduction in neurogenesis led to impaired spatial learning ability which was assessed by the Morris water maze test (Chapter 3). Consistent with the hippocampal results, in the other neurogenic regions in the brain (the SVZ-OB system), miR-21 KO mice showed reduced differentiation of NPCs both *in vitro* and *in vivo.* Additionally, differentiated neurons derived from miR-21 KO mice, *in vitro,* had reduced neurite outgrowth while SVZ-differentiated neurons derived from miR-21 OE mice demonstrated an increased neurite outgrowth (Chapter 5). The mechanism by which miR-21 mediated regulation of adult neurogenesis was suggested to be through the regulation of apoptotic genes, thus affecting the survival of immature neurons (Chapter 4). However, miR-21 can be involved in the regulation of other targets and investigation of other functional miR-21 targets can further elucidate its role in adult neurogenesis. These findings suggested an important role for miR-21 in neuronal survival and regenerative pathways, both mechanisms that are important

after traumatic brain injury or in neurodegenerative diseases. However, some findings still require more comprehensive investigations which I am going to address here.

Previous published studies indicated that miR-21 is expressed in neurons, astrocytes and microglia (Ge et al. 2014; Harrison et al. 2016b; Simeoli et al. 2017), suggesting that miR-21 may be involved in the regulation of their functions. Regulation of neurogenesis may be regulated directly by miR-21 that is present in the neurons, or indirectly via miR-21 expression in microglia and astrocytes, which can subsequently modulate the neurogenic niche. Cargo from extracellular vesicles can migrate from microglia and astrocytes to enter neurons to modulate their function (Frühbeis et al. 2013). Therefore, altered miR-21 expression can indirectly manipulate the niche to influence neurogenesis. To further pinpoint how miR-21 can regulate neurogenesis at the NPC stage or/and differentiated stage it will be vital to determine the cellular location of miR-21 such as co-localisation with NPC, neuronal, microglial or astrocytic markers. Unfortunately, localisation of miR-21 with microglia and neuronal markers, were not able to be detected. This was due to an absence of microglial and neuronal fluorescent staining, which can be explained by antigen loss due to multiple washes in the technique. Using immunostaining before ISH can be one of the suggested possible solutions. GFAP and miR-21 co-localisation was successful and this may be due to the abundance of astrocytes in the brain (Chapter 3, Figure 3-5). Additionally, localisation of miR-21 signal in the NPC was not investigated; this can be achieved using the *in situ* hybridisation of miR-21 signal with NPC markers such as nestin and Sox2. To

functionally dissect the contributions of cell-specific miR-21 to neurogenesis, further experimentation should be performed using cell-specific overexpression of knockdown of miR-21, for example by promoters that are specific for NPC (e.g. nestin), microglia (Iba1), astrocytes (GFAP) or neurons (eg. synapsin I). Cell-specific overexpression or knockdown of miR-21 can be achieved in targeted populations either by gene therapy/viral vector approaches or generating transgenic mice. This will enable us to ascertain the relative contributions of miR-21 to neurogenesis.

In relation to the above, the transgenic mice model used in this study was globally overexpressed and knocked out for miR-21 (Hatley et al. 2010). Despite the fact that miR-21 is an oncomiR, *CAG-miR-21* transgenic mice did not develop tumours and were undistinguished from their WT littermates; these observations are supported by other studies that have used the same mouse model (Hatley et al. 2010; McDonald et al. 2013; McDonald et al. 2015). To overcome the limitation of using global overexpression and knockdown of miR-21 and to account for any possible developmental effects, neurogenesis was analysed at an earlier time point at postnatal day 30. No differences were detected between the number DCX+ cells and volume of NeuN in the DG between transgenic mice compared to WT (Chapter 3), indicating that altered neurogenesis obtained in miR-21 transgenic mice are due to miR-21 effects in the adult stage and not due to earlier developmental effects. However, to better investigate miR-21 role in adult neurogenesis, other Cre recombinase models can be used, in which miR-21 can be

expressed specifically in NPCs in the adult brain upon tamoxifen injection such as *Nestin-Cre* (Kuo et al. 2006) or *GFAP-Cre* (Casper & McCarthy 2006).

MiR-21 expression in the adult brain is elevated post injury and in many cancer types. Upregulation of miR-21 is associated with increased cell proliferation and apoptotic resistance. In contrast, after traumatic brain injury miR-21 functions to increase astrocyte recovery to reduce lesion size and to reduce neuronal apoptosis (Han et al. 2014). MiR-21 has been found to regulate caspase-independent apoptosis pathway in neurodegenerative diseases (Sabirzhanov et al. 2012) and brain injury (Wu et al. 2013), by regulating the ratio of Bcl/Bax. Additionally, miR-21 has been reported to increase neurite outgrowth through the regulation of Pdcd4 following spinal cord injury (Jiang et al. 2017), and Spry2 following nerve injury (Strickland et al. 2011). This regenerative role of miR-21 can be applied as a possible therapy in neurogenerative diseases and after brain or spinal injury. A number of miRNAs with altered expression have been associated with neurogenerative diseases (reviewed in Shah et al. 2017). With respect to miR-21, increased expression was detected in the human immunodeficiency virus (HIV) dementia model (Yelamanchili et al. 2010). Prolonged NMDA receptor stimulation is an active excitotoxic process active in HIV, which can increase levels of miR-21. This increase in miR-21 can target the mRNA of myocyte enhancer factor 2C (MEF2C) and reduces its levels, leading to impaired learning and neuronal function. Although this suggest that increased miR-21 contributes to neuronal dysfunction and neurodegeneration, more studies are needed to understand miR-21 function in association with neurogenerative disorders.

Reduced hippocampal neurogenesis in miR-21 KO mice was linked to impairment in learning and memory in the Morris water maze (MWM) task (Chapter 3). Increased adult neurogenesis was found in the hippocampus of miR-21 OE although this did not reach significance when compared to the intact animals. This can be further investigated using other tasks such as Radial Maze (RAM), and working memory tasks including, delayed matching to sample (DMS) and delayed nonmatching to sample (DNMS) (Yau et al. 2015). Despite the relevance of MWM test to hippocampus dependent learning task and formation of spatial memories, the MWM test may not be sensitive enough to detect the contributions of adult born neurons to spatial learning (Bartsch 2012; Lieberwirth et al. 2016; Garthe & Kempermann 2013). Newly generated neurons are required for specific neuronal functions such as complicated spatial learning functional aspects rather than contribution to general hippocampus information processing in water maze test. Moreover, spatial performance in the water maze depends other structures beside the hippocampus including nucleus accumbens and caudate nucleus among others (Redish et al. 1997). Other tasks that can be used include open field exploration test, elevated plus-maze and elevated zero-maze to assess for the tendency of mice to explore novel environment and motor activity without affecting their motivation or memory (Bailey & Crawley 2009). Additionally, anxiety and depression related behavioural tests can be used to detect for possible impairment in miR-21 KO mice. The link between reduced adult hippocampal neurogenesis and depression like behaviour has been suggested in a number of studies (Jin et al. 2016; Cameron & Schoenfeld 2018). The glucocorticoid stress response, which is associated with depression and chronic stress, is increased in adult neurogenesis-deficient mice.

The hippocampus is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which controls the release of glucocorticoid (Roozendaal et al. 2001). Adult hippocampal neurogenesis found to reduce stress responses and depressive behaviour is linked to glucocorticoids, and mice deficient in adult neurogenesis demonstrated increased glucocorticoid response and showed depression like behaviour (Snyder et al. 2011). Anxiety and depression related behaviour can be investigated using sucrose intake test, forced swim test and tail suspended test to check for immobility time in mice. These tasks can further validate that miR-21 KO mice impaired learning in water maze test was due to reduced hippocampal associated learning and memory functions and not to their impaired motor function or anxiety.

In the OB, miR-21 KO mice had significant decrease in the number of differentiated neurons. Whether this will have an impact on functional behaviour remains to be investigated, as we did not perform OB-related behaviour tasks in this study. Future investigations could incorporate OB-behavioural tests to investigate OB function. The performance of adult and aged mice can also be compared to determine if the effects of miR-21 loss on olfactory functions are more dramatic in aged mice. It was reported that the proportion of GCs is increased with age (Imayoshi et al. 2008). The replacement rate of the pre-existing GCs in the OB by newly generated GCs increased with age, with the ratio of BrdU+/NeuN GCs after a time course of 6-18 months from 40% after 6 months to 60% after 12-18 months. Therefore, future tests of discrimination and memory of odours can be demonstrated using complicated odour discrimination tasks that require

discrimination between novel and recognisable odours to test for specific adult born interneuron impairment (Díaz et al. 2017). Despite the fact that there was only a slight reduction in adult born interneurons in miR-21 KO mice compared to WT mice, odour discrimination tests can be applied using several age groups to check if behavioural impairment associated with miR-21, if any, is going to increase with age.

In order to understand the regulatory mechanism of miR-21 function we investigated its expression in relation to other abundant miRNAs in the CNS. Recent studies using transcriptome analysis calculated the correlation for the expression of each miRNA with every other miRNA in 15 human tissue samples and 105 human cell lines, indicated a positive correlation between the expression of different miRNAs (Chaulk et al. 2016). However, in this study the expression levels for several neuron-enriched miRNAs such as miR-132, miR-134 and miR-212 were not changed with altered miR-21 expression, suggesting that there are no compensatory mechanisms or that miR-21 function is unrelated to the other miRNAs. This was observed in whole hippocampal tissue and specific isolation of DG tissue by microscopic microdissection or laser micro-dissection can enable GCL sorting to improve specificity and avoid expression dilution with other heterogenous cells in the hippocampus. RNA extracted from the GCL only can provide cell-specific mRNA for further analysis (Vincent et al. 2002). Additionally, virus can stereotaxically injected into the DG to label dividing cells, which can be either fluorescent activated cell sorted (FACS) or micro-dissected to quantify mRNAs and miRNAs expression. A retroviral vector with fluorescent

protein (FP) injected within the neurogenic regions can enable the infection of proliferating cells undergoing mitosis and thus, expressing the FP. This can allow the labelling of proliferating cells at different stages of differentiation (Ming & Song 2011).

A large number of studies have allocated several key pathways that are involved in the regulation of NPC proliferation, differentiation and survival, including Wnt/β-catenin pathway, Jak-Stat, MAPK and Glucocorticoid related signalling pathways (Aimone et al. 2014; Saaltink & Vreugdenhil 2014; Faigle & Song 2013). Several pathways and miR-21 targets were investigated to identify miR-21 regulatory pathways. Based on our immunohistochemistry (IHC) and western blot (WB) results, increased expression of the apoptotic marker Caspase-3 was detected in the DG of miR-21 KO mice, although this failed to reach significance (Chapter 3), suggesting that miR-21 may act as an anti-apoptotic factor in adult neurogenesis. This result was expected as the change in neuronal number was not through increased proliferation, but through cell survival. Our data indicated possible miR-21 regulation via the apoptotic marker Caspase-3. The expression of the pro-survival protein BCL2 in miR-21 OE mice was slightly upregulated, although again this failed to reach significance. The attempts to further validate results by the investigating the expression of the pro-apoptotic Bax protein were not successful (data not shown). However, it is unlikely that miR-21 directly regulates Caspase-3 as the UTR of Caspase-3 mRNA does not contain a miR-21 binding site. Other studies have demonstrated that the anti-apoptotic role of miR-21 is regulated by the activation of the PTEN/PI3K-Akt signalling pathway

(Zhou et al. 2010; Ren et al. 2010; Ge et al. 2014; Han et al. 2014), however in this study, there was no evidence of PTEN/Akt regulation by miR-21 as the protein levels of PTEN/AKT were unchanged in transgenic mice. Despite the investigation of several signalling pathways, our attempts to identify the modulated signalling pathway, was not conclusive.

Bioinformatics analysis predicted a number of possible target genes that are regulated by miR-21. The use of multiple target prediction tools enables the selection of common targets, which by their analysis, indicated possible involvement in regulation of apoptosis (Chapter 4). Using three different bioinformatics prediction tool, 76 genes were common in the three databases. Analysis of the 76 genes revealed that some of the well-known miR-21 targets that have been identified by a number of studies including, *Pdcd4, Pten, Bcl2* (Ou, Li & Kang 2014; Talotta et al. 2009; Wang et al. 2017; Sims et al. 2017) were absent, indicating that prediction programs may not incorporate all experimentally derived possibilities of miRNA:mRNA interactions. Further, only 7 genes were common utilising experimentally validated programmes, which highlights the need for experimental validation to identify functional targets.

It has been reported that miRNA regulated function was attributable to decreased mRNA levels, and result in decreased protein production. However, some miRNA targeting, mostly occurs at the translational repression level (Lim et al. 2005b; Guo et al. 2010).Therefore, to ensure that miR-21 binding to its target is reflected in functional interaction, crosslinking immunoprecipitation (CLIP)-seq is suggested

as a future experiment to validate true miRNA:target binding (Thomson et al. 2011; Ule et al. 2003). In the CLIP-seq method, hippocampus is dissected from transgenic mice and WT mice and miRNA:mRNA interactions within the RISC complex are locked by UV cross-linking following which the target mRNAs are isolated through a series of immunoprecipitation (using Argonaute antibody to pull down RISCs) and molecular biology steps and a library is prepared for next generation sequencing. This technique can not only validate functional mRNA targets, it may also reveal novel targets of miR-21.

In conclusion, this dissertation has uncovered a new role for miR-21 in regulating neurogenesis in the adult mouse brain. Certain aspects of the work require further investigation, for example the dissection of cell-specific effects of miR-21 and deciphering the exact components of the signalling pathways that miR-21 activates to mediate survival of the NPCs. The study of miR-21 expression with associated functional pathways is important to better understand its role in adult neurogenesis and cognitive functions. From the wider perspective, this information will also add to the body of knowledge on the role of miRNAs in brain function. Furthermore, understanding the molecular mechanisms underlying lineage determination can provide new theopoetical approach to prevent age-dependent loss of neurogenesis. This knowledge can be applied in the future to develop potential therapies for age-related neurodegeneration as well as neural disorders such as traumatic brain injuries.

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Appendix

Targets predicted by TargetScan, miRDB, microT-CDS v. 0.5.

Gene	**Description**
Scml2	Sex comb on midleg-like 2 (Drosophila)
Tgfbi	Transforming growth factor, beta induced; Plays a role in cell adhesion
Fam46a	Family with sequence similarity 46, member A
Gpr64	G protein-coupled receptor 64; Orphan receptor. Could be involved in a signal transduction pathway controlling epididymal function and male fertility. May regulate fluid exchange within epididymis
Ntf3	Neurotrophin 3; Seems to promote the survival of visceral and proprioceptive sensory neurons
Zfp367	Zinc finger protein 367; Transcriptional activator. Isoform 1 may be involved in transcriptional activation of erythroid genes (By similarity)
Arhgap24	Arhgap24 - Rho GTPase activating protein 24; involved in cell polarity, cell morphology and cytoskeletal organization. Controls actin remodelling by inactivating Rac downstream of Rho leading to suppress leading edge protrusion and promotes cell retraction to achieve cellular polarity. Able to suppress RAC1 and CDC42 activity in vitro.
Armcx1	Armadillo repeat containing, X-linked 5

Epha4	Epha4 - Eph receptor A4; Receptor tyrosine kinase which binds membrane-bound ephrin family ligands residing on adjacent cells, leading to contact-dependent bidirectional signalling into neighbouring cells.
Peli1	Pellino 1; E3 ubiquitin ligase catalyzing the covalent attachment of ubiquitin moieties onto substrate proteins (By similarity). Involved in the TLR and IL-1 signalling pathways via interaction with the complex containing IRAK kinases and TRAF6. Mediates 'Lys- 63'-linked polyubiquitination of IRAK1 allowing subsequent NF- kappa-B activation
Tiam1	T cell lymphoma invasion and metastasis 1; Modulates the activity of RHO-like proteins and connects extracellular signals to cytoskeletal activities. Activates RAC1, CDC42, and to a lesser extent RHOA. Required for normal cell adhesion and cell migration (By similarity). Affects invasiveness of T-lymphoma cells
Map3k1	Mitogen-activated protein kinase kinase kinase 1; Component of a protein kinase signal transduction cascade. Activates the ERK and JNK kinase pathways by phosphorylation of MAP2K1 and MAP2K4. Activates CHUK and IKBKB, the central protein kinases of the NF-kappa-B pathway
Caskin1	CASK interacting protein 1; May link the scaffolding protein CASK to downstream intracellular effectors
Pbrm1	Pbrm1 - Polybromo 1; Involved in transcriptional activation and repression of select genes by chromatin remodelling (alteration of DNA-nucleosome topology). Acts as a negative regulator of cell proliferation
Fasl	Fas ligand (TNF superfamily, member 6); Cytokine that binds to TNFRSF6/FAS, a receptor that transduces the apoptotic signal into cells. May be involved in cytotoxic T-cell mediated apoptosis and in T-cell development.
Zfp704	Zfp704 - Zinc finger protein 704
Pcsk6	Pcsk6 - Proprotein convertase subtilisin/kexin type 6
Elf2	Elf2 - E74-like factor 2; Probably transcriptionally activates the LYN and BLK promoters and acts synergistically with RUNX1 to transactivate the BLK promoter
Thrb	Thrb - Thyroid hormone receptor beta; Nuclear hormone receptor that can act as a repressor or activator of transcription.
Stag2	Sgol1 - Shugoshin-like 1 (S. pombe); Plays a central role in chromosome cohesion during mitosis by preventing premature dissociation of cohesion complex from centromeres after prophase, when most of cohesion complex dissociates from chromosomes arms. Essential for proper chromosome segregation during mitosis and this function requires interaction with PPP2R1A.
Rasgrp1	RAS guanyl releasing protein 1; Functions as a calcium- and diacylglycerol (DAG)- regulated nucleotide exchange factor specifically activating Ras through the exchange of bound GDP for GTP. Activates the Erk/MAP kinase cascade. Regulates T-cell/B-cell development, homeostasis and differentiation by coupling T-lymphocyte/B-lymphocyte antigen receptors to Ras. Regulates NK cell cytotoxicity and ITAM- dependent cytokine production by activation of Ras-mediated ERK and JNK pathways (By similarity).

Spry2	Sprouty homolog 2 (Drosophila); May function as an antagonist of fibroblast growth factor (FGF) pathways and may negatively modulate respiratory organogenesis
Pcbp1	Pcbp1 - poly(rC) binding protein 1; Single-stranded nucleic acid binding protein that binds preferentially to oligo dC
Jph1	Junctophilin 1; Junctophilins contribute to the formation of junctional membrane complexes (JMCs) which link the plasma membrane with the endoplasmic or sarcoplasmic reticulum in excitable cells. Provides a structural foundation for functional cross-talk between the cell surface and intracellular calcium release channels.
Ubr3	Ubiquitin protein ligase E3 component n-recognin 3; E3 ubiquitin-protein ligase which is a component of the N-end rule pathway.
Ski	Ski - Ski sarcoma viral oncogene homolog (avian); May play a role in terminal differentiation of skeletal muscle cells but not in the determination of cells to the myogenic lineage. Functions as a repressor of TGF-beta signalling
Pja2	Praja 2, RING-H2 motif containing; Has E2-dependent E3 ubiquitin-protein ligase activity.
Ccl1	Chemokine (C-C motif) ligand 1; Cytokine that is chemotactic for neutrophils
Nfia	Nuclear factor I/B; These proteins are individually capable of activating transcription and replication
Pcbp2	poly(rC) binding protein 2; Single-stranded nucleic acid binding protein that binds preferentially to oligo dC. Major cellular poly(rC)-binding protein. Binds also poly(rU).
Plekha1	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1
Gramd3	GRAM domain containing 3
Edrf1	RIKEN cDNA 2700050L05 gene; Transcription factor involved in erythroid differentiation. Involved in transcriptional activation of the globin gene (By similarity)
Yap1	Yes-associated protein 1; Transcriptional regulator which can act both as a coactivator and a corepressor and is the critical downstream regulatory target in the Hippo signalling pathway that plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis.
Jag1	Jagged 1; Ligand for multiple Notch receptors and involved in the mediation of Notch signalling. May be involved in cell-fate decisions during hematopoiesis. Inhibits myoblast differentiation (By similarity). May regulate fibroblast growth factor-induced angiogenesis
Klf6	Kruppel-like factor 6; Transcriptional activator. Binds a GC box motif. Could play a role in B-cell growth and development (By similarity)
Glcci	Glucocorticoid induced transcript 1
Hipk3	Homeodomain interacting protein kinase 3; Serine/threonine-protein kinase involved in transcription regulation, apoptosis and steroidogenic gene expression. Phosphorylates JUN and RUNX2. Seems to negatively regulate apoptosis by promoting FADD phosphorylation.

Tmem170	Tmem170 - Transmembrane protein 170; Acts as a regulator of endoplasmic reticulum (ER) and nuclear envelope (NE) morphogenesis. Affects the ratio between tubular ER and ER sheets by promoting sheet formation at the expense of tubules.
Spry1	Sprouty homolog 1 (Drosophila); May function as an antagonist of fibroblast growth factor (FGF) pathways and may negatively modulate respiratory organogenesis
Chic1	Chic1 - Cysteine-rich hydrophobic domain 1
Pfkm	Pfkm - Phosphofructokinase, muscle; Catalyses the phosphorylation of D-fructose 6-phosphate to fructose 1,6-bisphosphate by ATP, the first committing step of glycolysis
Gid4	GID complex subunit 4, VID24 homolog (S. cerevisiae)
Smad7	Smad7 - SMAD family member 7; Antagonist of signalling by TGF-beta (transforming growth factor) type 1 receptor superfamily members; has been shown to inhibit TGF-beta (Transforming growth factor) and activin signalling by associating with their receptors thus preventing SMAD2 access. Functions as an adapter to recruit SMURF2 to the TGF-beta receptor complex.
Reck	Reck - Reversion-inducing-cysteine-rich protein with kazal motifs; Negatively regulates matrix metalloproteinase-9 (MMP-9) by suppressing MMP-9 secretion and by direct inhibition of its enzymatic activity. RECK down-regulation by oncogenic signals may facilitate tumor invasion and metastasis. Appears to also regulate MMP-2 and MT1-MMP, which are involved in cancer progression (By similarity)
Suz12	Suppressor of zeste 12 homolog (Drosophila); Polycomb group (PcG) protein. Component of the PRC2/EED- EZH2 complex, which methylates 'Lys-9' (H3K9me) and 'Lys-27' (H3K27me) of histone H3, leading to transcriptional repression of the affected target gene.
Nifb	Nfib - Nuclear factor I/B; Recognizes and binds the palindromic sequence 5'- TTGGCNNNNNGCCAA-3' present in viral and cellular promoters and in the origin of replication of adenovirus type 2. These proteins are individually capable of activating transcription and replication
Yod1	Yod1 - YOD1 OTU deubiquitinating enzyme 1 homologue (S. cerevisiae); Hydrolase that can remove conjugated ubiquitin from proteins and participates in endoplasmic reticulum-associated degradation (ERAD) for misfolded lumenal proteins.
Mef2c	Mef2c - Myocyte enhancer factor 2C; Transcription activator which binds specifically to the MEF2 element present in the regulatory regions of many muscle- specific genes. Controls cardiac morphogenesis and myogenesis and is also involved in vascular development. May also be involved in neurogenesis and in the development of cortical architecture. Plays an essential role in hippocampal-dependent learning and memory by suppressing the number of excitatory synapses
Acat1	- acetyl-Coenzyme A acetyltransferase 1; Plays a major role in ketone body metabolism
Wwp1	Wwp1 - WW domain containing E3 ubiquitin protein ligase 1; E3 ubiquitin-protein ligase which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly

	transfers the ubiquitin to targeted substrates. Ubiquitinates and promotes degradation of SMAD2 in response to TGF-beta signalling, which requires interaction with TGIF (By similarity).
Alx1	ALX homeobox 1; Sequence-specific DNA-binding transcription factor that binds palindromic sequences within promoters and may activate or repress the transcription of a subset of genes. Most probably regulates the expression of genes involved in the development of mesenchyme-derived craniofacial structures.
Kdm7a	Jhdm1d - Jumonji C domain-containing histone demethylase 1 homolog D (S. cerevisiae); Histone demethylase required for brain development. Specifically demethylates dimethylated 'Lys-9' and 'Lys-27' (H3K9me2 and H3K27me2, respectively) of histone H3 and monomethylated histone H4 'Lys-20' residue (H4K20Me1), thereby playing a central role in histone code.
Ehd1	Ehd1 - EH-domain containing 1; ATP- and membrane-binding protein that controls membrane reorganization/tubulation upon ATP hydrolysis. Acts in early endocytic membrane fusion and membrane trafficking of recycling endosomes. Recruited to endosomal membranes upon nerve growth factor stimulation, indirectly regulates neurite outgrowth.
Ccl22	Chemokine (C-C motif) ligand 22; Chemotactic for activated T-lymphocytes. May play an important role in the collaboration of dendritic cells and B- lymphocytes with T-cells in immune responses
Srl	Sarcalumenin; May be involved in the regulation of calcium transport
Il21	Il21 - Interleukin 21; Cytokine with immunoregulatory activity. May promote the transition between innate and adaptive immunity. Induces the production of IgG(1) and IgG(3) in B-cells. May play a role in proliferation and maturation of natural killer (NK) cells in synergy with IL15. During T-cell mediated immune response may inhibit dendritic cells (DC) activation and maturation
Pik3r1	Pik3r1 - Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha); Binds to activated (phosphorylated) protein-Tyr kinases, through its SH2 domain, and acts as an adapter, mediating the association of the p110 catalytic unit to the plasma membrane. Plays an important role in signalling in response to FGFR1, FGFR2, FGFR3, FGFR4, KITLG/SCF, KIT, PDGFRA and PDGFRB. Likewise, plays a role in ITGB2 signalling (By similarity).
Adtrp	Adtrp - Androgen dependent TFPI regulating protein; Regulates the expression and the cell-associated anticoagulant activity of the inhibitor TFPI in endothelial cells (in vitro)
Arhgef12	Arhgef12 - Rho guanine nucleotide exchange factor (GEF) 12; May play a role in the regulation of RhoA GTPase by guanine nucleotide-binding alpha-12 (GNA12) and alpha-13 (GNA13).
Dkk2	- Dickkopf homolog 2 (Xenopus laevis); Antagonizes canonical Wnt signalling by inhibiting LRP5/6 interaction with Wnt and by forming a ternary complex with the transmembrane protein KREMEN that promotes internalization of LRP5/6. DKKs play an important role in vertebrate development, where they locally inhibit Wnt regulated processes such as, somitogenesis and eye formation. In the adult, Dkks are implicated in bone formation and bone disease, cancer and Alzheimer disease

Krit1	KRIT1, ankyrin repeat containing; Component of the CCM signalling pathway which is a crucial regulator of heart and vessel formation and integrity. Negative regulator of angiogenesis. Inhibits endothelial proliferation, apoptosis, migration, lumen formation and sprouting angiogenesis in primary endothelial cells. Promotes AKT phosphorylation in a NOTCH-dependent and independent manner and inhibits ERK1/2 phosphorylation indirectly through activation of the DELTA-NOTCH cascade.
Mprip	Myosin phosphatase Rho interacting protein; Targets myosin phosphatase to the actin cytoskeleton. Required for the regulation of the actin cytoskeleton by RhoA and ROCK1. Depletion leads to an increased number of stress fibers in smooth muscle cells through stabilization of actin fibers by phosphorylated myosin. Overexpression of MRIP as well as its F- actin-binding region leads to disassembly of stress fibers in neuronal cells
Ppp1r3a	Ppp1r3a - Protein phosphatase 1, regulatory (inhibitor) subunit 3A; Seems to act as a glycogen-targeting subunit for PP1. PP1 is essential for cell division, and participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. Plays an important role in glycogen synthesis but is not essential for insulin activation of glycogen synthase
Fubp1	Far upstream element (FUSE) binding protein 1; Regulates MYC expression by binding to a single-stranded far-upstream element (FUSE) upstream of the MYC promoter. May act both as activator and repressor of transcription (By similarity)
Slc30a10	Slc30a10 - Solute carrier family 30, member 10; Plays a pivotal role in manganese transport. Manganese is an essential cation for the function of several enzymes, including some crucially important for the metabolism of neurotransmitters and other neuronal metabolic pathways
Ube2d3	Ube2d3 - Ubiquitin-conjugating enzyme E2D 3; Accepts ubiquitin from the E1 complex and catalyzes its covalent attachment to other proteins. In vitro catalyzes 'Lys- 11'-, as well as 'Lys-48'-linked polyubiquitination.
Pag1	Phosphoprotein associated with glycosphingolipid microdomains 1; Negatively regulates TCR (T-cell antigen receptor)- mediated signaling in T-cells and FCER1 (high affinity immunoglobulin epsilon receptor)-mediated signaling in mast cells. Promotes CSK activation and recruitment to lipid rafts, which results in LCK inhibition. Inhibits immunological synapse formation by preventing dynamic arrangement of lipid raft proteins.
Rspo2	R-spondin 2 homolog (Xenopus laevis); Activator of the canonical Wnt signalling pathway by acting as a ligand for LGR4-6 receptors. Upon binding to LGR4-6 (LGR4, LGR5 or LGR6), LGR4-6 associate with phosphorylated LRP6 and frizzled receptors that are activated by extracellular Wnt receptors, triggering the canonical Wnt signalling pathway to increase expression of target genes. Also regulates the canonical Wnt/beta-catenin-dependent pathway and non-canonical Wnt signalling by acting as an inhibitor of ZNRF3, an important regulator of the Wnt signalling pathway.

Map2k3	Mitogen-activated protein kinase kinase 3; Dual specificity kinase. Is activated by cytokines and environmental stress in vivo. Catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in the MAP kinase p38. Part of a signalling cascade that begins with the activation of the adrenergic receptor ADRA1B and leads to the activation of MAPK14
Slmap	Slmap - Sarcolemma associated protein; May play a role during myoblast fusion
Rab11a	RAB11a, member RAS oncogene family; The small GTPases Rab are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes. Rabs cycle between an inactive GDP-bound form and an active GTP-bound form that is able to recruit to membranes different set of downstream effectors directly responsible for vesicle formation, movement, tethering and fusion. That Rab regulates endocytic recycling. Acts as a major regulator of membrane delivery during cytokinesis.
Dusp8	Dusp8 - Dual specificity phosphatase 8; This protein shows both activity toward tyrosine-protein phosphate as well as with serine/threonine-protein phosphate
Crebrf	RIKEN cDNA A930001N09 gene; Acts as a negative regulator of the endoplasmic reticulum stress response or unfolded protein response (UPR). Represses the transcriptional activity of CREB3 during the UPR. Recruits CREB3 into nuclear foci (By similarity)
Klhl42	Klhdc5 - Kelch domain containing 5; Substrate-specific adapter of a BCR (BTB-CUL3-RBX1) E3 ubiquitin-protein ligase complex required for mitotic progression and cytokinesis. The BCR(KLHL42) E3 ubiquitin ligase complex mediates the ubiquitination and subsequent degradation of KATNA1